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ANAEROBIC BIODEGRADATION OF BENZENE UNDER ENVIRONMENTAL CONDITIONS OF SUB-ARCTIC GROUNDWATERS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy
in
Environmental Engineering

By

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Fairbanks, Alaska

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By

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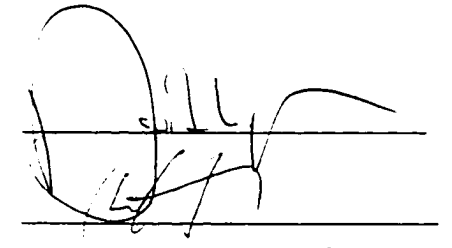

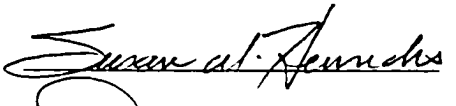

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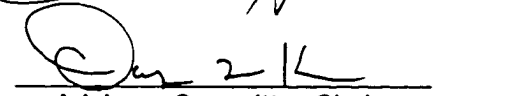

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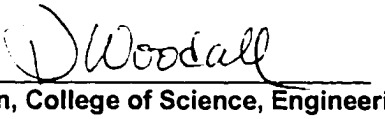

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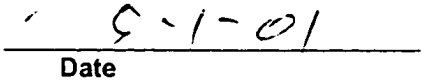

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ABSTRACT

This research was conducted to detect and understand the potential for anaerobic degradation of benzene in a sub-arctic aquifer environment. The major hypothesis tested in this dissertation is that indigenous microorganisms from anaerobic groundwater wells contaminated with BTEX (benzene, toluene, ethylbenzene, xylenes) can use benzene as a carbon or energy source under conditions favoring iron- and sulfate-reduction and methanogenesis. To test this hypothesis microcosms were prepared and incubated under anaerobic conditions, with either hematite (Fe_2O_3) or sodium sulfate (Na_2SO_4) added to permit growth of iron and sulfate reducing bacteria. Several concentrations of benzene were used as a substrate, and the microcosms were incubated at temperatures of 21 °C and 4 °C. Cell counts were done and some characteristics of the bacteria, such as shape, gram staining, and spore formation were determined.

The aromatic hydrocarbon, benzene, was anaerobically transformed under both psychrophilic (4 °C) and mesophilic (21 °C) conditions. The temperature and substrate concentration played a role in determining the rate at which benzene was biodegraded under conditions favorable to iron-reducing, sulfate-reducing, or methanogenic bacteria.

The rate of degradation under conditions favoring methanogenesis was faster than that under iron and sulfate-reducing conditions at 4 °C, but

not at 21 °C. In general, at 21 °C the biodegradation of benzene was faster in the presence of an exogenous electron acceptor. Benzene degraded completely under iron and sulfate-reducing conditions but did not degrade completely in most of the microcosms under methanogenic conditions. Phenol and was detected as intermediates for this transformation under all conditions.

Benzene degradation rates were independent of the electron acceptor. This suggests that removal of benzene was initiated via fermentation reactions occurring under all conditions. Addition of a small concentration of sulfate or ferric as electron acceptors made degradation more complete.

The *in vitro* research conducted on the anaerobic biodegradation of benzene provided new insight into the anaerobic degradation processes that occur at cold temperatures associated with sub-arctic environments. It also provided insights of the roles different types of microorganisms may play in the natural attenuation of contaminants.

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CHAPTER ONE

INTRODUCTION

1.1 Problem and Background

Major contamination of groundwater in the United States arises from spillage of gasoline or other petroleum-based fuels and from leakage of these same fuels from underground fuel storage tanks. Feliciano estimated in 1984 that between 75,000-100,000 such tanks in the United States were leaking. The most water-soluble components of petroleum contaminants are the aromatic compounds, benzene, toluene, ethylbenzene, and xylene (BTEX). Although pump-and-treat remediation methods are used to clean contaminated groundwater at some sites, alternative remediation methods are needed due to the high cost of pump-and- treat systems and their ineffectiveness in removing residual contamination sorbed to soil particles. Pump-and-treat systems are used most often to contain contaminant plumes within a set boundary. An alternative, cost- effective remediation strategy is *in situ* bioremediation.

Indigenous microorganisms in BTEX-contaminated groundwater can use the contaminants as a source of carbon, energy, and building blocks for new bacterial cells. When aerobic bacteria use the contaminants in this way, they consume dissolved oxygen as a terminal electron acceptor, which often leads to anaerobic conditions. Anaerobic bacteria require alternative electron acceptors such as sulfate (SO_4^{-2}) and iron (Fe^{+3}). Study of the degradation of benzene under anaerobic and low temperature conditions is needed to better evaluate the

potential contribution of anaerobic bioremediation for contaminated site restoration.

Zobell and Agosti (1972) and Kerry (1990) found that psychrophilic microorganisms have the ability to degrade a variety of contaminants aerobically. The research of Edwards and Gribic`-Galic` (1992, 1994), Evans et al. (1991), and Haag et al. (1991) indicates that bioremediation of groundwater contaminated with aromatic hydrocarbons, including benzene, is possible using mesophilic anaerobic bacteria. Vogel and Gribic`-Galic` (1986) showed that the anaerobic oxidation of toluene and benzene was initiated by oxygen derived from ^{18}O-labeled water; then, toluene and benzene were also fermentatively oxidized with oxygen from ^{18}O-labeled water. Alternate electron acceptors, such as nitrate (Kuhn et al., 1985), ferric (Lovely and Lonergan, 1990), and sulfate (Edward et al., 1992; Reinhard et al., 1997) support microbial degradation of aromatic pollutants. However, anaerobic biodegradation of aromatic hydrocarbons at temperatures below 10 ^{o}C, in the presence of electron acceptors such as SO_4^{-2} and Fe^{+3}, has not been studied in detail previously.

1.2 Objectives

This research was conducted to detect and understand the potential for anaerobic degradation of benzene in a sub-arctic aquifer environment. The major hypothesis tested by this dissertation is that indigenous microorganisms from anaerobic groundwater wells contaminated with BTEX can acclimate, *in vitro,* using benzene as a

carbon and energy source under conditions favoring iron- and sulfate-reduction and methanogenesis. Experiments were conducted under iron- and sulfate-reducing and methanogenic conditions with incubation at temperatures of 21 °C and 4 °C.

The primary goal of this research was to obtain information about the biodegradation of benzene and other BTEX compounds in natural soil and groundwater systems under anaerobic conditions and at low temperatures. Understanding the steps in the transformation of benzene and other compounds could help engineers predict the fate of contaminants in soil and groundwater in cold regions. More specifically, the following were research objectives of this investigation:

1. Determine the degree of transformation and mineralization of benzene under anaerobic conditions and at temperatures of 4 °C and 21 °C.
2. Identify the role of microorganisms during mineralization of the BTEX compounds.
3. Understand the tolerance of the microorganisms for variations of environmental factors such as substrate concentration and availability of electron acceptors.
4. Partially characterize groups of microorganisms involved in the biodegradation.

1.3 Research Approach

The soil and groundwater in Operable Unit 5 (OU5) at Fort Wainwright, Alaska, have become contaminated with petroleum hydrocarbons and chlorinated solvents as the result of leakage from underground storage tanks and pipelines, accidental spills, and improper handling and disposal techniques. This site has an average annual ground temperature of 4 °C and, hence, offered the opportunity to collect bacteria living in this cold anaerobic environment and study BTEX degradation *in vitro*.

The approach to investigating the degree of transformation and mineralization of benzene under anoxic conditions and at temperatures of 4 °C and 21 °C was a combination of field and laboratory techniques. The methods used in this research are discussed in detail in Chapter 3. Existing chemical and microbial data collected by the United States Geological Survey and the Water and Environmental Research Center, University of Alaska Fairbanks, was used to select the sampling locations for the study. The concentration of the benzene, reduction of terminal electron acceptors, and production of intermediates are among the factors that were measured *in vitro* to detect the transformation and mineralization of benzene. An evaluation of the data discussed in Chapter 3 favored the selection of well FWM 6894 for sampling for this research.

Laboratory studies were conducted in the dark under anoxic conditions favoring iron- or sulfate- reduction or fermentation and methanogenesis. The

microorganisms were incubated at two different temperatures. In addition, the initial concentrations of the benzene substrate were varied.

The characterization of the microorganisms was also important to this research. The total number of bacteria (TNB) method described by Edwards and Gribic`-Galic` (1994) and Bratbak (1993) were used to measure the overall growth of the microorganisms. As described by Gerhardt et al. (1994), detection of the presence of microorganism spores and endospores by the Schaefffer-Fulton Endospore Method and capsules by the Dugid and Hiss staining method was performed. Using an epifluorescence microscope, as described by Bratbak (1993), the shape of the microorganisms was determined. The Gram staining method was used to identify the cells as Gram positive or Gram negative. Because proper preservation of cultures was important, the culture was maintained by regular subculture on nutrient media and by drying and deep freezing, following the methods described by Gerhardt et al. (1994) and Edwards and Gribic`-Galic`(1994).

1.4 Outline

This dissertation is a collection of six chapters that collectively address the hypothesis presented earlier. Each chapter was written as an independent unit. Chapter 1 introduces the problem, states the hypothesis and objectives and the general approach, and describes the outline of the dissertation. Chapter 2 is a general literature review of research done in the past related to aromatic hydrocarbon biodegradation. Chapter 3 is a description of the site, sampling, and

experimental design. Chapter 4 is a discussion of the anaerobic degradation of benzene under iron- and sulfate-reducing conditions at room temperature (21 °C) and at 4 °C. Chapter 5 is a discussion of the anaerobic degradation of benzene under conditions of methanogenesis at room temperature (21 °C) and at 4 °C. Chapter 6 is a discussion of the overall conclusions of this work and a summary of the major findings. Suggestions for future research are also given.

CHAPTER TWO

LITERATURE REVIEW

2.1 Bacteria and Biological Processes

Hydrocarbons are not only found in the immediate area of recent spills; chemical analysis has revealed the presence of both biogenic and petroleum-derived hydrocarbons in most soils and sediments (Giger and Blumer, 1974), irrespective of recent use history. Perhaps because of the widespread occurrence of natural analogs, microorganisms can use hydrocarbon contaminants as a source of carbon for the basic building blocks of new cell constituents and as a source of electrons and energy. The organic contaminant is oxidized, and the microorganisms grow and reproduce.

Many microorganisms use molecular oxygen as an electron acceptor under aerobic conditions. Other microorganisms, such as methanogens and sulfate- and iron-reducing bacteria, use electron acceptors including carbon dioxide, sulfate, or iron (III) under anaerobic conditions. The final products of these selected aerobic and anaerobic processes are summarized in Table 2.1. The compounds are arranged in order from most oxidized to most reduced. The tendency of a compound to accept or release electrons is expressed quantitatively by its reduction potential (E_o). More energy is available when oxygen is used than when another electron acceptor is used (Brock et al., 1994).

Table 2.1: Selected aerobic and anaerobic processes involved in the microbial metabolism of organic matter (modified fromThauer et al., 1977b).

Process	Electron Acceptor	Metabolic Products	Reduction Potential E_o' (V)
Aerobic Oxidation	O_2	CO_2, H_2O	+0.82
Iron Reduction	Fe^{3+}	CO_2, Fe^{2+}	+0.77
Sulfate Reduction	SO_4^{2-}	CO_2, H_2S	-0.217
Methanogenesis	CO_2	CO_2, CH_4	-0.24

In an oxygen-free environment, fermentation can take place. In fermentation, the microorganisms use organic substances as both electron donors and electron acceptors. The major end products of bacterial fermentation are propionic acid, formic acid, acetic acid, butyric acid, ethanol, butanol, isopropanol, 2, 3-butanediol, carbon dioxide, hydrogen, and methane (Cookson, 1995). Fermentation products can usually be biodegraded by other types of bacteria, converting them to carbon dioxide, methane, and water.

In certain situations, enzymes generated by an organism to degrade a primary substrate as a source of carbon and energy can also transform another, secondary substrate. This process is known as cometabolism. The degradation of the secondary substrate occurs only in

the presence of the primary substrate. Both primary and secondary substrates can compete for enzymes, causing inhibition or slowing of degradation. Maintaining a low primary substrate concentration can enhance the rate of cometabolism (McCarty, 1987).

When mixtures of hydrocarbon contaminants are present, competitive inhibition of the degradation of some compounds can result. Sometimes, there is no competition when different metabolic routes degrade the different aromatic hydrocarbons present (Law and Button, 1986). But in the contrary situation, if such aromatic substrates share the same metabolic routes, competitive inhibition can result and bacterial growth can be reduced (Gibson et al., 1968).

It was generally believed that aromatic compounds with no oxygen in their molecular structure would resist microbial attack and be recalcitrant under anaerobic conditions (Kazumi et al., 1997). However, recent work has shown that anaerobic degradation is possible. Factors that could be important for anaerobic degradation include the availability of other electron acceptors, nutrients, and substrates (Alexander, 1965).

In soil and fresh water, electron acceptors, such as oxidized nitrogen, sulfate, or oxidized metals like Fe (III) and Mn (IV), can serve as oxidants in anaerobic oxidation. In the above cases, some aromatic compounds can be completely oxidized to CO_2 (Lovely, 1991).

For fermentation of aromatic compounds to occur, a strong reducing agent such as hydrogen or formate is needed for the initial reduction of the aromatic ring (Fucus et al., 1994). Such reactions are energetically unfavorable under most conditions (Stull et al., 1969; Thauer et al., 1977a,b). These reactions become favorable in the presence of H_2-consuming organisms such as sulfate-reducing bacteria and methanogens. The products are CO_2 and H_2S or CO_2 and methane.

2.1.1 Methanogenesis

Methanogens belong to the kingdom Archaebacteria. They are widespread in strictly anaerobic environments. Methanogens can use only a limited range of substrates such as H_2, CO_2, acetate, and methyl groups of C-1 compounds (Fenchel and Finaly, 1995). Most molecules with more than two carbon atoms cannot be converted to methane by methanogenic bacteria. The exceptions are those with easily cleaved methyl groups such as those in methionine. Other fermentative bacteria must convert complex organic compounds to H_2, CO_2 and acetate before the methanogenic bacteria can convert them to methane. Fermentative and methanogenic bacteria can establish a syntrophic association, but neither of these groups alone can degrade complex organic compounds completely (Zender, 1978). Fermentative bacteria can hydrolyze high molecular weight substances, such as organic polymers, protein and lipids, into smaller compounds, which, in turn, are fermented to aliphatic

alcohols, acids, acetate, methanol, formate, or molecular hydrogen and CO_2 (Gribic`-Galic`, 1990). The fermentative bacteria will then transform these products to hydrogen and more oxidized products, such as acetate and CO_2 to obtain more energy. The hydrogen-scavenging methanogens will cleave the acetate into CO_2 and CH_4 and use the H_2 produced by fermenters as the electron donor to reduce CO_2 to methane. These reactions are thermodynamically favorable under an extremely low partial pressure of H_2. The methanogenesis in this case will drive the reaction forward by consuming H_2 (Strayer and Tiedje, 1978). Gribic`-Galic` (1990) summarized the transformation of organic polymers to methane, as illustrated in Figure 2.1.

2.1.2 Sulfate-reducing Bacteria

Sulfate-reducing bacteria belong to the kingdom Eubacteria and grow in strictly anaerobic environments. Sulfate-reducing bacteria use sulfate as an electron acceptor to oxidize organic substances and produce energy. Sulfate-reducing bacteria cannot degrade natural polymers, so they depend on fermentation products of other anaerobic bacteria. They can outcompete methanogens and acetogens for common substrates such as H_2 and acetate (Oremland and Taylor, 1978; Horikoshi and Grant, 1991; Gupta et al., 1994a).

Oremland and Marsh (1982) showed that both sulfate reduction and methanogenesis can occur concurrently if the groups utilize different

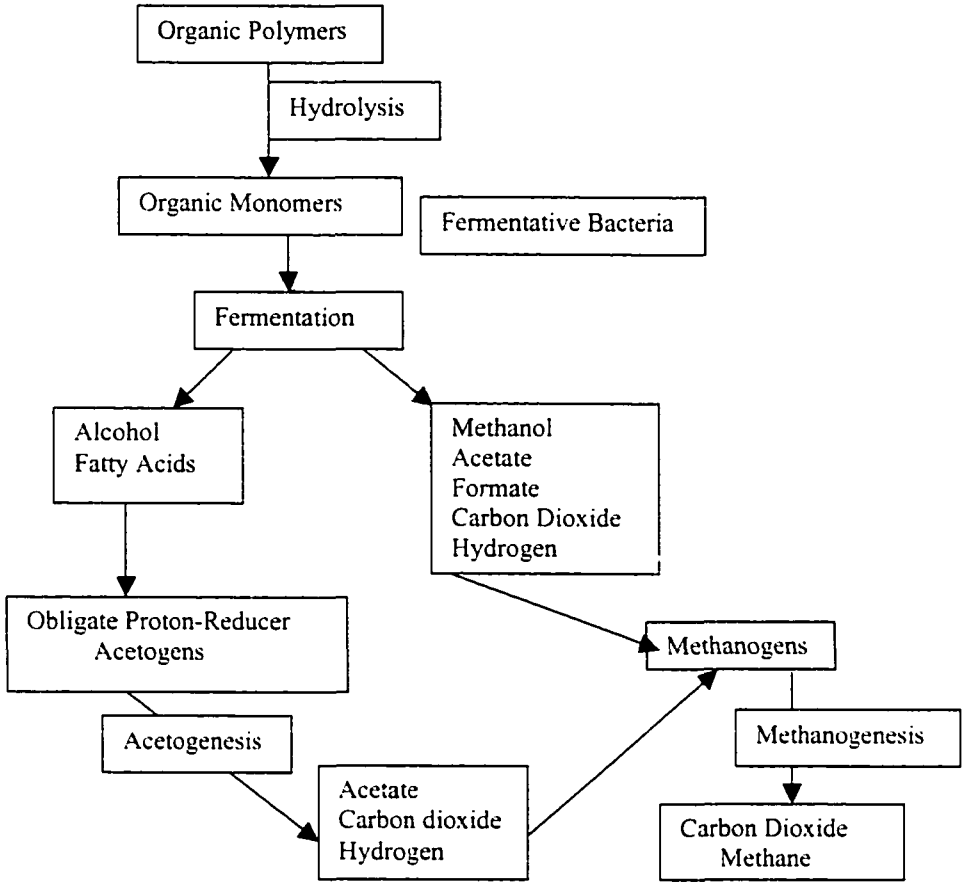

Figure 2.1: Microbial degradation of complex organic compounds under methanogenic conditions (modified from Gribic'-Galic', 1990).

electron donors or one group provides an electron donor needed by the other group. This relationship is called synergism. As in methanogenesis, the fermenters and the hydrogen-scavenging microorganisms, such as sulfate-reducing bacteria, can establish a syntrophic association. Sulfate-reducing bacteria can use acids, acetate, methanol, formate, and other substances as electron donors. The sulfate-reducing bacteria will also drive the fermentative reactions forward by consuming H_2. The end products are H_2S and CO_2 (Figure 2.2).

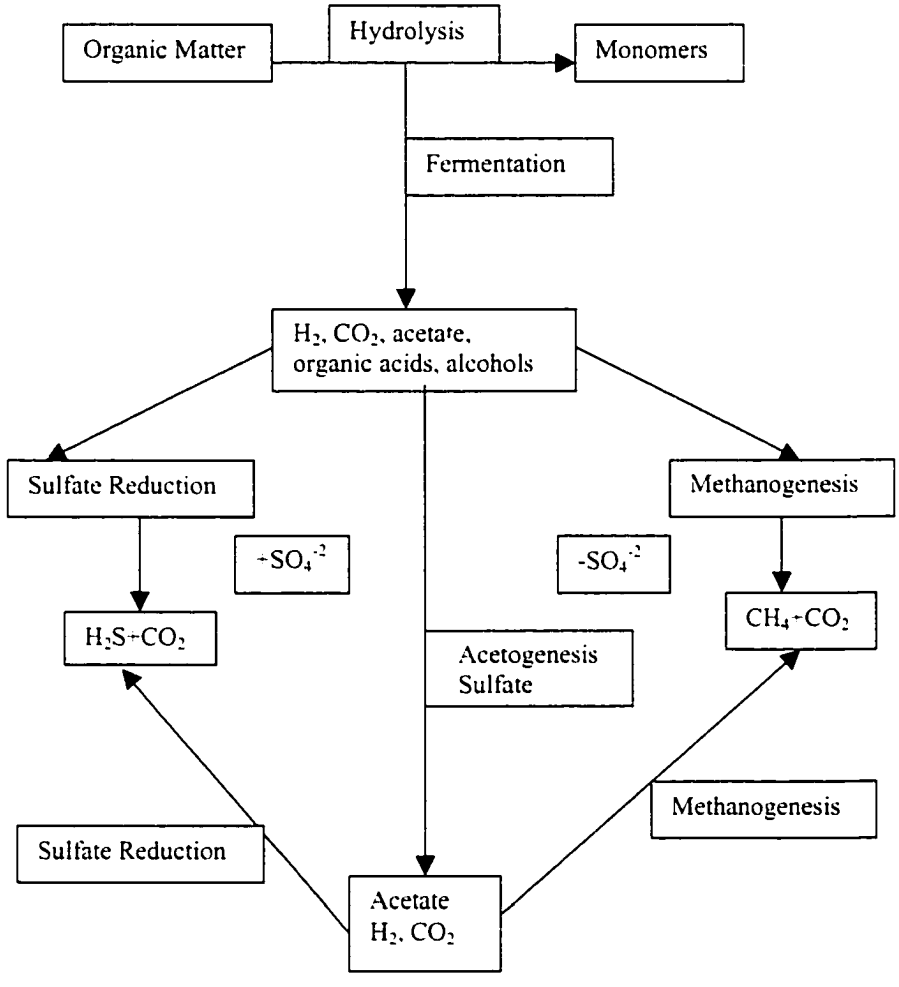

Figure 2.2: Microbial degradation of complex organic compounds under sulfate-reducing conditions (modified from Fauque and Barton, 1995).

2.1.3 Iron-reducing Bacteria

Iron-reducing microorganisms can outcompete sulfate reducers and methanogens for hydrogen and acetate (Lovely and Phillips, 1987b). The hydrogen partial pressure required by iron-reducing bacteria is lower than that under conditions where methanogenic and sulfate-reducing bacteria predominate. Also, the iron-reducing bacteria cause the concentration of H_2 and acetate to be so low, sulfate-reducing bacteria and methanogenesis are inhibited (Lovely, 1986a, b). Although the complete oxidation of fermentable substrates to CO_2 and the transfer of all the electrons to Fe (III) is thermodynamically favorable, only a minor portion of the electrons from fermentable substrates is transferred. Yet, iron-reducing bacteria are not excluded from metabolizing fermentable products and mineralizing organic matter with ferric iron as the sole electron acceptor (Lovely, 1986a, b). The end product of iron reduction is Fe (II).

2.1.4 Psychrophilic Microorganisms

Temperature plays an important and direct role in regulating the growth rate, enzyme activity, cell composition, and nutritional requirements of bacteria (Herbert, 1981). It also has an indirect role, through effects on ion transport and diffusion, osmosis, surface tension, and solubility of solute molecules (Openheimer, 1970). Forster (1987) is believed to be the first person to report the growth of bacteria at 0 °C in

marine fish. Later, bacteria that were able to grow at low temperatures were isolated from permanently cold environments, such as ocean waters (Garey and Waksman, 1934), polar soils (Straka and Stokes, 1960; Vishniac and Hempfling, 1979 a, b), Antarctic marine and fresh waters (Herbert and Bell. 1973; Tanner and Herbert, 1981), and cave systems (Gounot, 1973). Tanner and Herbert (1981) demonstrated at Signy Island in the maritime Antarctic that nutrient recycling was due to the activities of psychrophilic bacteria active at a temperature of 1 °C.

Microorganisms that grow at 0 °C or lower, and have an optimal growth temperature of 15 °C or less, are called psychrophilic bacteria (Brock et al., 1994). Organisms that grow at 0 °C but can tolerate temperatures between 20-40 °C are called psychrotolerant. Most of the psychrophilic bacteria are Gram-negative species (Herbert, 1981).

2.2 Energetics

In most cases, microbial communities oxidizing organic matter will primarily use the terminal electron acceptor, of those available, that yields the most energy from organic matter oxidation. The terminal electron acceptor of microbial mineralization is determined by the amount of free energy that a microorganism can obtain from coupling an oxidation reaction with the potential reduction reactions. Stoichiometry and standard free energies of reactions related to the metabolism of benzene are summarized in Table 2.2. The free energy for each reactant and

product is summarized in Table 2.3. Under anaerobic conditions, thermodynamic considerations suggest that electron acceptors such as iron would be favored over sulfate, which in turn is favored over carbon dioxide.

Table 2.2: Stoichiometry and an estimate of standard free energy of reactions at pH 7 related to the metabolism of benzene (Wiedemeier et al., 1995; Thauer et al., 1977b). This stoichiometry assumes no cellular mass is produced (modified from Wiedemeier et al., 1995).

Reaction	Reactants	Products	$\Delta G^{o'}$ (kcal/mole)
Aerobic respiration	$7.5\ O_2 + C_6H_6$	$6CO_2 + 3H_2O$	-817.136
Iron reduction	$H^+ + 1Fe^{+3} + C_6H_6$	$6CO_2 + Fe^{2+} + 3.5H_2O$	-801.811
Sulfate reduction	$7.5\ H^+ + 3.75\ SO_4^{2-} + C_6H_6$	$6CO_2 + 3.75H_2S + 3H_2O$	-57.911
Methanogenesis	$4.5\ H_2O + C_6H_6$	$2.25CO_2 + 3.75CH_4$	-32.261

Table 2.3: Standard Free energy of formation for various compounds (1 molar, 25 °C, pH 7) (modified from Thauer et al., 1977b).

Compound	$\Delta G^{o'}$ (formation) (kcal/mol)
Benzene (l)	29.756
Phenol	-11.38
Methane	-12.14
CO_2 (g)	- 94.26
HCO_3^-	-140.26
Water (l)	- 56.69
H^+(aq) at pH 7	- 9.67
H_2 (g)	0
SO_4^{2-}	-177.34
SH^- (aq)	2.88
H_2S (aq)	- 8.02
Fe^{+3}(aq)	-1.1
Fe^{+2}(aq)	-18.85
O_2	6.9

2.3 Previous Laboratory and Field Studies

2.3.1 Aerobic Biodegradation of Aromatic Compounds

Aerobic degradation of aromatic compounds has been studied in detail (Evans, 1963). Molecular oxygen is required to prepare the benzene ring for cleavage. Dagley (1984), who studied aerobic aromatic hydrocarbon biotransformation, found that the addition of oxygen by oxygenase leads to biodegradation of the aromatic ring. Catechol was formed as the result of the ring cleavage. Barker et al. (1987) observed that the aerobic metabolism of aromatic hydrocarbons in aquifers was difficult to maintain. As oxygen was consumed due to aerobic biodegradation in the plume, anaerobic conditions were established and inhibited the metabolism of benzene, toluene, and xylene. In such situations, there was a need for continuous sparging, continuous monitoring, and installation of wells to deliver oxygen. Major et al. (1988) recommended adding peroxide, to increase subsurface oxygen concentrations. However, such addition produced metal oxides that precipitated and reduced the hydraulic conductivity of the aquifer.

2.3.2 Anaerobic Biodegradation of Aromatic Compounds

Anaerobic biotransformation of aromatic compounds has been shown to occur under methanogenic conditions (Healy and Young, 1978, 1979; Healy et al., 1980; Kaiser and Hanselmann, 1982; Horowitz et al., 1983; Gribic`-Galic` and Young, 1985), during denitrification (Taylor et al.,

1970; Williams and Evans, 1975; Braun and Gibson, 1984), under iron and sulfate reducing conditions (Edwards and Gribic`-Galic`, 1994; Lovely et al., 1995), and under unspecified reducing conditions (Hallas and Alexander, 1983; Boyd and Shelton, 1984; Liu et al., 1984). In these studies, the aromatic compounds had oxygen-containing substituents, e.g., phenol, benzoate, and catechol.

Tarvin and Buswell (1934) confirmed anaerobic degradation of aromatic compounds such as benzoate. Later, Clark and Fina (1952) and Nottingham and Hungate (1969) confirmed that benzoate was degraded to CO_2 and CH_4. Dutton and Evans (1969) suggested that methanogenic fermentation of aromatic compounds could be accomplished via reduced intermediates. Taylor et al. (1970) were the first to report the isolation of a pure culture of a microorganism (Pseudomonas PN-1) that could oxidize aromatic compounds aerobically in the presence of oxygen and anaerobically in the presence of nitrate (NO^-_3).

The first indication of degradation of non-oxygenated aromatic compounds was from studies related to the Amoco Cadiz spill in 1979 (Cookson, 1995). Subsequent studies found that denitrifying bacteria degraded aromatic compounds that lacked oxygen-containing substituents (Vogel and Gribic`-Galic`, 1986; Wilson et al., 1986). Major et al. (1988) studied the potential for remediation by adding nitrate as an electron acceptor to an aquifer contaminated with benzene, toluene, and xylene.

Degradation of BTEX during denitrification was confirmed, resulting in the accumulation of nitrous oxide instead of nitrogen gas.

The pathway for the anaerobic breakdown of the aromatic ring is distinct and independent of the aerobic pathway. The first step in the degradation of a non-oxygenated aromatic compound is conversion of the compound to an oxygenated form. In an anaerobic environment, oxygen is first incorporated from water into the aromatic structure via hydroxylation (Vogel and Gribic`-Galic`, 1986). In the case of benzene, the degradation is initiated by ring oxidation, resulting in the formation of phenol in mixed cultures. Once the initial oxidation occurs the anaerobic degradation follows pathways similar to those of phenol, as discussed by Evans (1977), Gribic`-Galic` and Vogel (1987), and Lovely and Lonergan (1990) (Figures 2.3, 2.4, and 2.5). Suidan et al. (1981, 1988) also evaluated the degradation of phenol under anaerobic conditions. Caldwell and Suflita (2000) observed the degradation of benzene under methanogenic, iron- and sulfate-reducing conditions at room temperature. Phenol and benzoate were found as intermediates of anaerobic benzene decay (Figure 2.6).

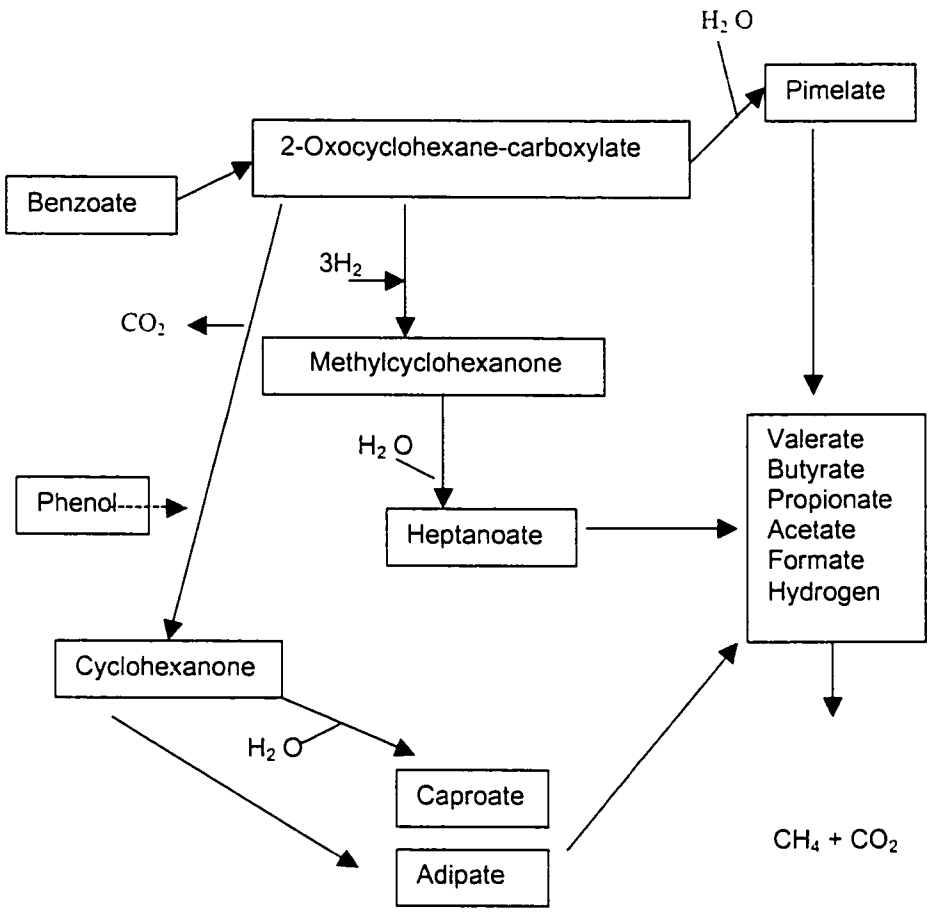

Figure 2.3: Probable pathway of the fermentation of benzoate and phenol by adapted bacterial consortia from a variety of methanogenic ecosystems (modified from Evans, 1977).

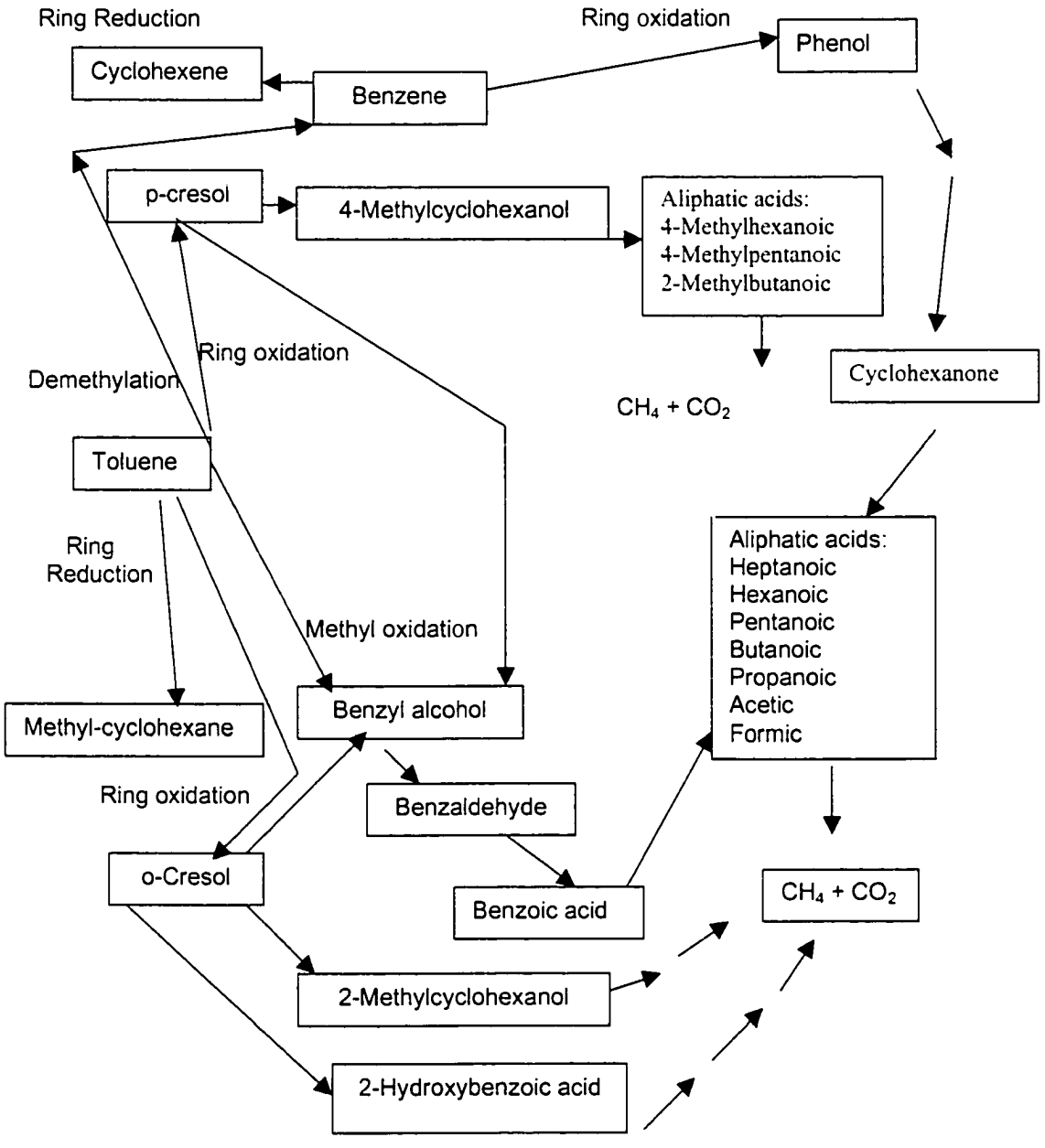

Figure 2.4: Tentative sequences of anaerobic toluene degradation by mixed methanogenic cultures. All the compounds shown were detected and identified by GC-MS (modified from Gribic`-Galic` and Vogel, 1987).

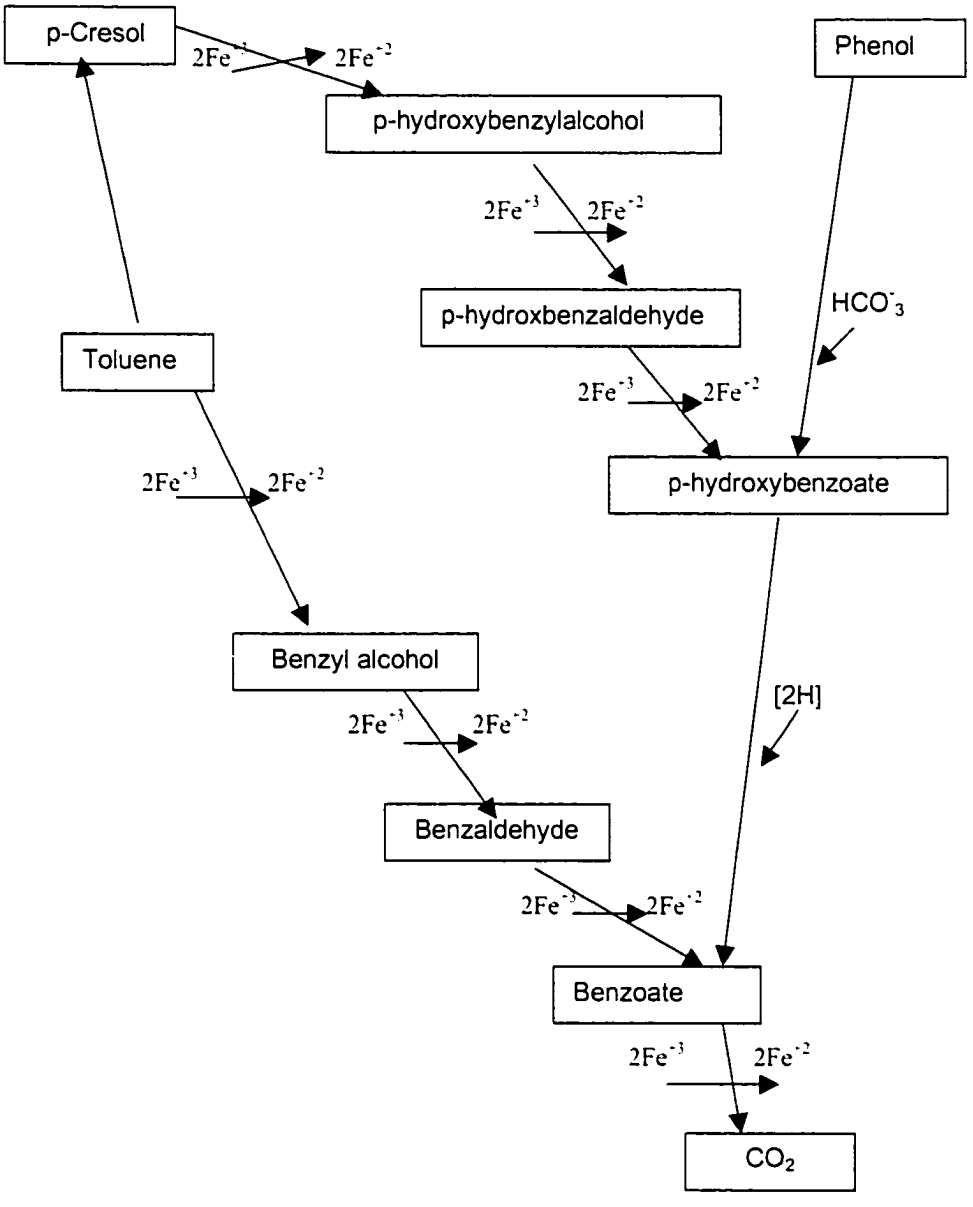

Figure 2.5: Potential pathways for the oxidation of toluene, p-cresol, and phenol coupled to Fe (III) reduction (modified from Lovely and Lonergan, 1990).

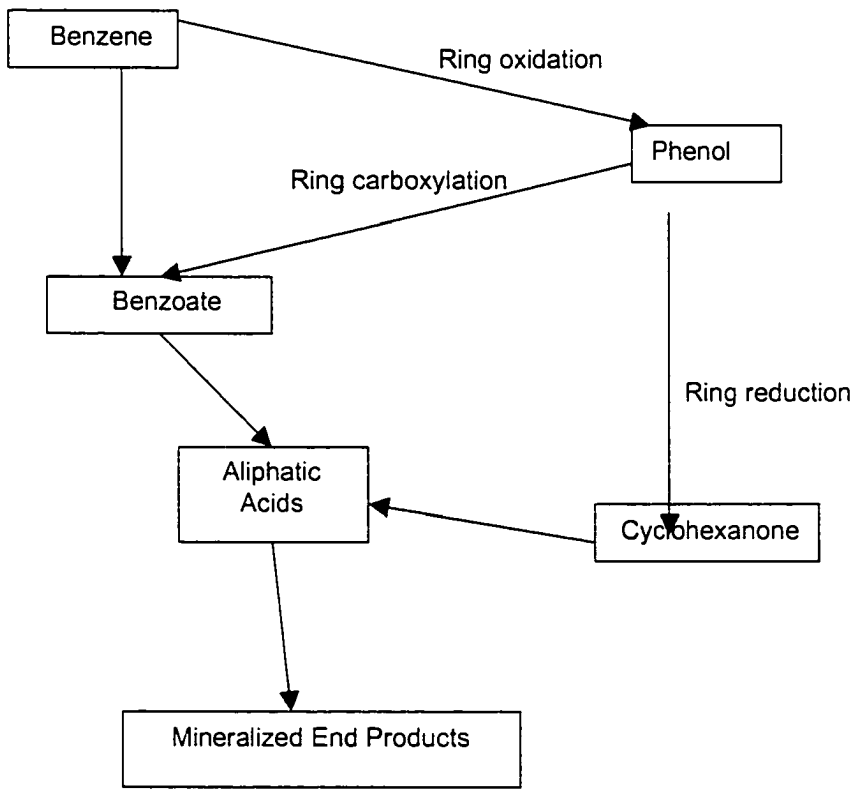

Figure 2.6: Proposed pathway for anaerobic biodegradation of benzene that proceeds through benzoate (modified from Caldwell and Suflita, 2000).

Rapid anaerobic biodegradation of toluene and xylene has been found in a denitrifying aquifer column (Kuhn et al., 1985; Zeyer et al., 1986). Kuhn et al. (1987) evaluated the use of different electron acceptors, NO_3^-, NO_2^- and N_2O, for the anaerobic oxidation of toluene and m-xylene. These substrates were mineralized in the presence of nitrate or nitrous oxide in an anaerobic laboratory aquifer column operated under continuous flow conditions. Such biodegradation was inhibited when nitrate was replaced by nitrite or when molecular oxygen was added. The m-xylene-adapted microorganism in the aquifer column degraded toluene and some other aromatic compounds, but was unable to metabolize certain aromatic compounds such as benzene. Kuhn et al. (1987) suggested that benzoate was the intermediate formed during the anaerobic metabolism of toluene. Mineralization was confirmed by trapping $^{14}CO_2$ evolved from ^{14}C-ring-labeled substrates.

For another study, aquifer-derived microorganisms were obtained from cresol-contaminated sediment (Edwards and Gribic`-Galic`, 1994). The samples were collected down-gradient of the contamination source, in an actively methanogenic sandy zone of the aquifer. Under strictly anaerobic conditions, an enrichment was prepared using periodic addition of toluene and o-xylene. Toluene and o-xylene were degraded as predicted by the Monod kinetic model and were completely mineralized to carbon dioxide, methane, and cell mass. The acclimation period was 100-

120 days for toluene and 200-255 days for o-xylene. When the concentration of the substrates exceeded 700 μM for toluene and 1,800 μM for o-xylene, degradation ceased. Degradation of toluene and o-xylene was inhibited when oxygen, nitrate, or sulfate was used as the electron acceptor. Degradation was also inhibited when additional substrates were added such as acetate, H_2, propionate, methanol, acetone, glucose, amino acids, fatty acids, peptone, and yeast extract. The toluene degradation activity was retained after starvation for one year. Also, the degradation activity was decreased by 25% when the temperature was decreased from 35 °C to 20 °C. The culture did not degrade benzene, m-xylene, p-xylene, ethylbenzene, or naphthalene.

Wilkes et al. (1996) isolated two different types of marine sulfate-reducing bacteria that depleted toluene, o- and m-xylene, o- and m-ethyltoluene, m-propyltoluene, and m-cymene from crude oil at varying rates under mesophilic conditions. The organisms that were isolated exhibited a high substrate specificity.

2.3.3 Biodegradation of Benzene

Marr and Stone (1961), Gibson et al. (1968), and Axell and Geary (1975) studied aerobic biodegradation of benzene. These studies identified two pathways, their intermediates, and the characteristics of the enzyme systems that were involved in the biodegradation. Catechol was formed initially, and it was further transformed by either catechol 1,2

dioxgenase or catechol 2,3 dioxygenase (Smith, 1994). Many bacterial strains that can grow on benzene have been isolated (Shirari, 1986; Van den Twell et al., 1988; Winstanley et al., 1987). Winstanley et al. (1987) reported that *Acinetobacter calcoaceticus* - RJE74 carries a large plasmid (pWW174) that encodes the enzymes for the catabolism of benzene via the catechol 1,2- dioxygenase, the first identified plasmid encoding enzymes for anaerobic aromatic hydrocarbon degradation.

In the environment, microorganisms usually are confronted with a mixture of aromatic hydrocarbons. Arvin et al. (1989) observed that the presence of either toluene or xylene stimulated the biodegradation of benzene, but the presence of both at the same time had an inhibitory effect. Alvarez and Vogel (1991) showed that benzene was degraded in the presence of toluene and p-xylene by *Arthrobacter* species, but biodegradation of benzene and toulene were retarded in the presence of p-xylene in *Pseudomonas* species.

In the past, many attempts to show that benzene could be biodegraded in an anaerobic environment failed. Schink (1985) observed no degradation of benzene under methanogenic conditions. Using enrichment cultures from anaerobic sewage sludge, freshwater sediments, and marine sediments. Kuhn et al. (1987) demonstrated failure to anaerobically oxidize benzene by a m-xylene-adapted microorganism, using NO_3^-, NO_2^- and N_2O as electron acceptors.

Wilson and Kampbell (1992) conducted field studies on a plume resulting from contamination of groundwater with gasoline in Michigan. As BTEX compounds were degraded, there was a decrease in dissolved oxygen followed by anaerobic degradation supported by nitrate and sulfate. After the depletion of nitrate and sulfate, methanogenesis occurred, and methane production increased, as BTEX compounds were further degraded. Total organic carbon was reduced but benzene failed to degrade. Another field study of *in situ* biodegradation of BTEX under anaerobic conditions showed a strong relationship between the disappearance of some aromatic hydrocarbons from contaminated groundwater and the appearance of associated metabolic byproducts. However, benzene and ethylbenzene concentrations did not decrease significantly during the experiment (Beller et al., 1995).

Methanogenic, sulfate, iron, manganese- and nitrate- reducing conditions were used to examine the transformation of the aromatic hydrocarbons toluene, benzene, and naphthalene (Langenhoff et al., 1995). Toluene was degraded with all of the electron acceptors tested. The majority of the naphthalene was transformed under sulfate-reducing conditions, and it was partly transformed under manganese- and nitrate-reducing conditions. Naphthalene concentration did not change under methanogenic and iron-reducing conditions. Benzene also did not

decrease in concentration within a period of 375-525 days under any of the conditions tested (Langenhoff et al., 1995).

Although anaerobic biodegradation of benzene was not detected in many earlier studies, some experiments have shown this can occur. Vogel and Gribic`-Galic` (1986) experimented with methanogenic cultures. They showed that the anaerobic oxidation of toluene and benzene was initiated by oxygen derived from ^{18}O-labeled water. The methanogenic culture was obtained from a stable methanogenic consortium that was degrading ferulic acid to CO_2 and methane. The initiation of the experiment with ^{18}O-labeled water was done after maintaining the cultures on toluene and benzene as the sole carbon and energy sources for one year. Gas chromatography-mass spectrometry was used to detect the ^{18}O-labeled metabolites. The cresol from toluene was up to 8% ^{18}O-labeled and the phenol from benzene contained up to 9% ^{18}O-labeled compound. This experiment was the first to report the introduction of oxygen from water into toluene and benzene under anaerobic conditions.

In another study, Gribic`-Galic` and Vogel (1987) used mixed methanogenic cultures to degrade toluene and benzene. The gas production and the ^{14}C-activity distribution in the products were monitored over a period of 60 days. A low percentage of radiolabelled $^{14}CO_2$ from ring-labeled toluene or benzene and a high percentage of $^{14}CO_2$ from the

methyl group-labeled toluene were produced. This is an indication that there was an incomplete conversion of the ring carbon to CO_2 and almost a complete conversion of the methyl group to CO_2 rather than methane. It was suggested that during the anaerobic transformation, toluene and benzene undergo fermentation, and the substrates are partially reduced and partially oxidized. Catechol, an intermediate typical of aerobic degradation of benzene, was not found in the anaerobic transformation. During anaerobic degradation, the non-oxygenated benzene was converted to an oxygenated form, phenol, by ring oxidation. Evans (1977) and Gribic`-Galic` and Vogel (1987) proposed a pathway for transformation of phenol under methanogenic conditions.

Biodegradation of toluene, benzene, and xylene was observed in a contaminated aquifer when nitrate was added as an electron acceptor (Major et al., 1988). Cozzarelli et al. (1990) reported that anaerobic bioattenuation of alkylbenzene, toluene, o-xylene, and benzene occurred in a plume of contamination from a spill of crude oil in Minnesota. Toluene and o-xylene disappeared without a lag period, suggesting that the microorganisms had already adapted to degrade these compounds. Ethylbenzene degradation began after the disappearance of toluene and o-xylene. Benzene was degraded without a lag period in the presence of o-xylene and toluene. The rate of benzene degradation decreased when these compounds disappeared. The presence of methane in the

groundwater and the accumulation of long-chain volatile fatty acids indicated biodegradation. In the same study, the presence of phenol and aromatic organic acids was related to the biodegradation of benzene and allkylbenzene in a methanogenic and iron-reducing environment.

Alvarez et al. (1991) used aquifer material that had been exposed to benzene, toluene, and xylene. Mixed and pure cultures were prepared in serum bottles, sealed with Teflon-lined caps and aluminum crimps, and incubated upside down in the dark at 25 ^{o}C. Biodegradation of different combinations of benzene, toluene and p-xylene was observed in the presence of mixed cultures, and pure cultures of *Pseudomonas* sp. strain CFS-215 and *Arthrobacter* sp. strain HCB that were isolated from the same site. Benzene and p-xylene degradation was enhanced in the presence of toluene in *Pseudomonas* sp. strain CFS-215 incubations. The rate of benzene degradation increased and the lag period decreased from 6 days to 1 day when toluene was used as the sole co-substrate. On the other hand, when p-xylene was present, the degradation of benzene was inhibited. There was no degradation when benzene and p-xylene were the only compounds present. Benzene degradation by *Arthrobacter* sp. strain HCB was also dependent on toluene and xylene. Toluene was only degraded in the presence of benzene, and p-xylene was not significantly degraded. The lag period for benzene was 2 days and was not affected by the presence of toluene or p-xylene alone. When both

toluene and p-xylene were present concurrently, the lag period of benzene increased to 4 days. The presence of benzene also enhanced the degradation of p-xylene and toluene. Degradation of benzene was fastest when it was present alone.

Edwards and Gribic`-Galic` (1992) observed complete mineralization of benzene to carbon dioxide when sulfate was provided as a potential electron acceptor. Edwards amended subsurface sediment from Seal Beach, California, with benzene in an anaerobic environment. Microcosms were prepared under sulfate-reducing conditions and amended with benzene at concentrations varying from 40 to 200 μM. The microcosms were incubated in the dark inside an anaerobic chamber at room temperature. Benzene was degraded in active microcosms, but the electron acceptor was not determined.

Laboratory and pilot-scale studies were conducted by the Naval Facilities Engineering Service Center (NFESC) on the anaerobic degradation of BTEX compounds in groundwater. The study took place at Naval Weapons Station in Seal Beach, California, at an unleaded gasoline spill site (U.S. Environmental protected Agency, 1994). In the laboratory, soil and groundwater from Seal Beach were amended with nutrients, carbon sources and electron acceptors under anaerobic conditions. Under sulfate-reducing conditions bacteria degraded toluene, benzene, m-xylene, and p-xylene. Ethylbenzene was degraded only under nitrate-

reducing conditions. Three bioreactors were inserted into the ground at the site. Each had a column filled with contaminated soil and groundwater was pumped from the bottom of the column up through the soil. The first column maintained sulfate-reducing conditions, since the groundwater contains naturally high levels of sulfate. In the second column, nitrate-amended groundwater was pumped up, and the third column was used as a control. Anaerobic degradation of toluene, m-xylene, and p-xylene occurred in the first and second columns. In the second column ethylbenzene was anaerobically degraded. This suggested that a combination of electron acceptors might be necessary to initiate and accelerate the degradation of BTEX.

Reinhard et al. (1997) evaluated the biodegradation of another gasoline-contaminated aquifer at Seal Beach, California under nitrate and sulfate-reducing conditions. With no addition of electron acceptors, there was a slow transformation of toluene and xylene. With added nitrate, toluene, ethylbenzene, and m-xylene were transformed without a lag phase within 10 days, while o-xylene was transformed within 72 days. With added sulfate, toluene, m-xylene, and o-xylene were completely removed within 50 days, while ethylbenzene was removed within 60 days. Benzene removal was limited under sulfate-reducing conditions.

Sulfate was the terminal electron acceptor in another study of anaerobic benzene biodegradation (Lovely et al., 1995). Highly reduced

sediments were incubated under anaerobic conditions. Benzene was directly oxidized to carbon dioxide, without any detection of intermediates within 55 days. The initial benzene concentration added was 1 μM.

Anderson et al. (1998) evaluated the potential anaerobic degradation of benzene by iron-reducing bacteria at different sites. Oxidation of benzene failed at most of the sites except for one site in Bemidji, Minnesota. This site differed from the other sites by having more microorganisms of the family Geobacteraceae.

A recent study by Kazumi et al. (1997) observed the microbial degradation of benzene under methanogenic, sulfate-, iron-, and nitrate-reducing conditions at room temperature and 30 °C. Benzene was degraded by the first three types of bacteria but failed to degrade under denitrifying conditions. The source of the sediment was a factor in determining the success of benzene biodegradation.

2.3.4 Biodegradation of Hydrocarbons at Low Temperatures

Many studies have reported that the biodegradation of petroleum by marine bacteria is temperature dependent. Atlas and Bartha (1972) added fresh crude oil to seawater collected from the New Jersey coast in September (temperature 17.5 °C) and December (temperature 7.5 °C). Phosphorus and nitrogen were added to the seawater. Incubation was at 5, 10, 15, and 20 °C. The percentages of the added oil degraded to CO_2 after 60 days were 8, 31, 46, and 46 for the September water sample and

20, 37, 46, and 46 for the December water sample. There was an increase of psychrophilic hydrocarbon-degrading bacteria in the December seawater, with an indication that the bacteria adapted to survive at low temperature. In another experiment done by Loynachan (1978) at 10 °C, stirring and fertilization enhanced mineralization of a mixture of Prudhoe Bay crude oil (Alaska) and soil. Aerobic bacteria were active shortly after oil addition and were followed by population increases of bacteria growing anaerobically.

Kerry (1990) suggested that there was a potential for degrading hydrocarbons at low temperatures when other conditions were favorable. He isolated various bacteria from petroleum-contaminated soils from Vesfold Hills and MacRobertson Land, Antarctica. Incubation was at 3 °C for six months, with occasional exposure to room temperatures and light. Four of the isolated colonies were active at temperatures below 5 °C; two *Corynebacterium* species, one *Flavobacterium* species and P138 (Family Enterobacteriaceae) could utilize hydrocarbons as a sole carbon source. P140 W (*Corynebacterium) and* P138 (Enterobacteriaceae) were isolated. Activity was observed in the aqueous phase at all temperatures between 1 °C and 28 °C. The activity of utilizing hydrocarbons for P140 was 75% and for P138 was 50% at 1 °C.

Lovely and Lonergan (1990) isolated a pure culture of a dissimilatory iron-reducing organism (GS-15) which oxidized toluene

under anaerobic conditions; Fe (III) oxide was the sole electron acceptor. Toluene was the sole electron donor and was completely oxidized to CO_2. Incubation occurred at 30 °C in the dark in anaerobic pressure tubes bubbled with N_2-CO_2 (80:20) gas and sealed with thick butyl rubber stoppers and aluminum crimp seals. No accumulation of intermediates occurred during degradation of toluene. In another experiment, they anaerobically incubated a pure culture of the Fe (III)-reducing bacterium GS-15 at a temperature of 9 °C for two months with sediments from an aquifer contaminated with crude oil in Bemidji, Minnesota. The accumulation of Fe (II) was observed when toluene, phenol, benzoate, and p-cresol were degraded as the sole substrates.

Bradley and Chapelle (1995) collected sediments from a 5 °C petroleum hydrocarbon-contaminated aquifer in Adak, Alaska. The aerobic mineralization of toluene was observed and compared to that of sediments contaminated with JP-4 jet fuel from Hanahan, South Carolina, incubated at a higher temperature, 20 °C. The hydrocarbon degradation was not depressed at the low temperature. On the contrary, the microbial community adapted to the cold temperature and used the hydrocarbon as a carbon and energy source. The results suggested an alternative clean up method, in-situ bioremediation, at cold temperature sites.

2.3.5 Biodegradation of Benzene at Low Temperatures

Recently, there have been studies of the anaerobic biodegradation of benzene over a range of temperatures. Beller et al. (1991) prepared microcosms and an enrichment of sediments collected from aquifer soils from Pensacola, Florida that had been stored for 2 years at 4 °C. The samples were obtained from an actively methanogenic, sandy zone of the aquifer. Experiments were prepared under strictly anaerobic conditions in screw-cap bottles that were sealed with Minert PTFE valves. The microcosms were initially fed with toluene, benzene, o-xylene, p-xylene, and ethylbenzene. Toluene started degrading after 100-120 days while o-xylene degraded after 200-225 days. The culture degraded toluene faster at 35 °C than at 20 °C. The culture was more active at pH 6 than at pH 7 and inactive at pH 8. Benzene, ethylbenzene, and p-xylene were not degraded.

In another study, non-contaminated aquifer material and material contaminated with JP-4 jet fuel was incubated in an anaerobic glove box and amended with nitrogen, nutrients, and aromatic hydrocarbons under a nitrogen atmosphere at 12 °C. In both contaminated and non-contaminated core materials, degradation occurred for several aromatic compounds. Benzene, ethylbenzene, and o-xylene were not significantly degraded within a 6-month time period. It is possible that recalcitrant compounds may need an extended adaptation period. When benzene

and m-xylene were individually tested in uncontaminated material, they slowed the rate of denitrification; Hutchins (1991) and Barbaro et al. (1992) concluded, in a field and laboratory experiment, that benzene was recalcitrant to biodegradation in the presence of nitrate at 10 °C.

Baedecker et al. (1993) observed that the degradation of hydrocarbons in a crude oil contaminated field near Bemidji, Minnesota, affected the concentration of oxidized and reduced aqueous species in the anoxic part of the contaminated plume. In anaerobic microcosm experiments conducted under field conditions at 9 °C, 10 μM of benzene, toluene, and naphthalene were added and Fe and Mn were the dominant electron acceptors. The reduction of the electron acceptors coincided with a degradation of 98% of the benzene within 125 days and the toluene within 45 days. Methane was not observed, probably because a small concentration of hydrocarbons was used. The results agreed with the field observation that methanogenesis developed after Fe and Mn reduction.

Hunt et al. (1994) conducted research at a petroleum-contaminated aquifer in North Carolina, where sulfate and iron, with negligible dissolved oxygen, dominated the plume electron acceptors. Redox potentials were -100 to -200 mV due to intrinsic biodegradation. Columns, including biologically active and abiotic controls, were inserted in the aquifer and filled with aquifer material and anaerobic groundwater containing BTEX. All columns were monitored on a monthly basis. Benzene and m- and p-

xylene decreased significantly after a period of 121 days. After an initial lag of 85 to 121 days m- and p-xylene decreased. Toluene decreased with no apparent lag time.

In the laboratory, multiple replicate microcosms were prepared with no headspace. Each contained blended aquifer sediment and groundwater under anaerobic conditions at the ambient groundwater temperature of 16 ^{o}C. The samples were monitored for ethylbenzene on a monthly basis for 400 days. Degradation of m-xylene occurred without a lag period, but the rate declined once toluene loss began after a 22 day acclimation period. The rate of m-xylene degradation increased again when toluene and o-xylene were below 20 μg/L. Benzene began to biodegrade after the depletion of xylene. The concentration of benzene was below 10 μg/L at the final sampling (403 days). Ethylbenzene loss was minimal.

In both *in-situ* columns and laboratory microcosms degradation of BTEX was obvious. The monitoring time was less for *in-situ* columns, which makes it difficult to compare the decay rates between in-situ and laboratory results.

Borden et al. (1997) evaluated the anaerobic biodegradation of benzene, toluene, ethylbenzene, and xylene isomers in two petroleum-contaminated aquifers at the ambient groundwater temperature of 16 ^{o}C. They used laboratory microcosms and in-situ test chambers. The

materials were collected from the middle and end of plumes in Sleeping Bear Dunes National Lakeshore, Michigan, and Rock Point, North Carolina. The first site consisted of glacial outwash with a combination of coarse sand and gravel with calcium carbonate fragments. In the field, toluene was biodegraded first and then ethylbenzene and xylene isomers. There was no evidence of benzene biodegradation. In the laboratory, toluene biodegraded under methanogenic conditions after a 60 to 246-day lag period. There was no evidence of benzene, ethylbenzene, and xylene biodegradation.

At the second site, the soils consisted of gray and green fine sand overlain by silts, clay, and clayey sand. All BTEX components biodegraded in the mid-plume microcosms under ambient anaerobic conditions. Under iron-reducing conditions, m -xylene biodegraded first, followed by toluene, o-xylene, and benzene. There was no evidence of biodegradation of the compounds in the source area microcosms. In the end-plume microcosms biodegradation varied, but slowed or stopped at a detectable level of BTEX.

2.4 Summary

Aromatic hydrocarbon degradation has been previously observed under both aerobic (Evans, 1963) and anaerobic conditions (Wilson et al. 1986; Vogel and Gribic`-Galic`, 1986; Langenhoff et al., 1995). The pathway for the anaerobic breakdown of the aromatic ring is distinct and

independent of the aerobic pathway. In an anaerobic environment, oxygen is first incorporated from water into the aromatic structure via hydroxylation. In the case of benzene, the degradation is initiated by ring oxidation resulting in the formation of phenol (Vogel and Gribic`-Galic`, 1986). It was suggested that during the anaerobic transformation, benzene undergoes fermentation and the substrates are partially reduced and partially oxidized (Gribic`-Galic` and Vogel, 1987).

Anaerobic biotransformation of benzene can occur with a variety of electron acceptors, such as iron (Anderson et al., 1998) and sulfate (Lovely et al., 1995), and under methanogenic conditions (Vogel and Griic`-Galic`, 1986), or non-specified reducing conditions (Edwards and Gribic`-Galic`, 1992). The fermenters and the hydrogen-scavenging microorganisms, methanogenic and sulfate-reducing bacteria, can establish syntrophic associations. Sulfate-reducing bacteria can outcompete methanogens for common substrates. Iron-reducing microorganisms can outcompete sulfate reducers and methanogens for hydrogen. Such relationships can play a role in determining the fate of benzene.

Complete mineralization of benzene to carbon dioxide was observed when sulfate was provided as a potential electron acceptor, but this study did not investigate whether other electron acceptors were present in the tested sediments (Edwards and Gribic`-Galic`, 1992).

Sulfate was clearly the terminal electron acceptor in a study by Lovely et al. (1995). Benzene was directly oxidized to carbon dioxide without any detection of intermediates within 55 days. Apparently, any intermeidiates produced were rapidly consumed and did not accumulate.

Anaerobic microcosm experiments conducted under field conditions at several temperatures greater than 9 °C indicated the potential degradation of benzene (Baedecker et al., 1993; Hunt et al., 1994). Low temperature does not necessarily prevent the anaerobic degradation of aromatic hydrocarbons in general, but anaerobic benzene biodegradation at temperatures less than 9 °C has not been observed. In addition, evaluation of anaerobic degradation of benzene at a variety of different sites is essential to understand variations in the activities of bacteria because of differing environmental conditions, soil types and climate. Previous studies did not associate the disappearance of benzene with factors other than biodegradation, such as adsorption, diffusion, evaporation, and chemical reactions.

Studies done in the past did not systematically investigate a variety of electron acceptors and the degradation of benzene at both high and low temperature under the same conditions. Among the environmental factors that could affect benzene biodegradation under anaerobic conditions are temperature, nutrient concentrations, and substrate concentration. Better understanding of the range of environmental conditions conducive to the

biodegradation of benzene could help to predict the fate of hydrocarbon contamination in soil and groundwater in cold regions.

CHAPTER THREE

FIELD SAMPLING AND LABORATORY SET UP

3.1 Introduction

A set of experiments was conducted to measure the anaerobic degradation of benzene in a simulated sub-arctic environment. The sampling procedure followed, materials used, experimental setup and methods employed to monitor the degradation of benzene are described in the following sections. Standard methods were used for the quantitative measurement of benzene and intermediate products of benzene degradation. All the methods used were applied to three types of microcosms, those supporting iron-reducing bacteria, sulfate-reducing bacteria, and methanogenic bacteria, unless otherwise specified. Additional studies on chemicals leaching from the butyl rubber stoppers were performed.

3.2 Site Description

Aquifer materials were obtained from Operable Unit 5 (OU5), East section, Former Quartermaster's Fueling System Source Area (east QFS Area), at the east side of building 1060, Fort Wainwright, Alaska. (See Appendix A). Fort Wainwright is located in the continental climate zone of interior Alaska. The surface soils at Fort Wainwright consist of wind blown silt, and the subsurface soil is predominantly glacial outwash consisting of

silt, sands, and gravel (See Appendix B). Liquid contaminants released into the subsurface system migrate downward through the unsaturated silt, sand, and gravel to the shallow water table.

Underground storage tanks (UST) and aboveground storage tanks (AST) of gasoline and diesel, and a network of aboveground and buried fuel piping connecting the fueling system components, existed in the East QFS Area (See Appendix C, historical aerial photograph). An investigation done by CH2M Hill, Microwell, and Harding Lawson Associates (Harding Lawson, 1996) identified a plume of petroleum hydrocarbons and solvents in groundwater, including gasoline range organics (GRO) and diesel range organics (DRO) such as benzene, trichloroethene (TCE), and dichloroethene (DCE). Microbiological data indicated the presence of diesel and gasoline degraders and other heterotrophic microorganisms. Microbiological data were collected and analyzed by the University of Alaska Fairbanks and the U.S. Geological Survey, Fairbanks, Alaska (See Appendix D).

3.2.1 Field Groundwater Sampling

Groundwater samples were collected from well FMW 6894, on the east side of building 1060 in the East QFS Area, Fort Wainwright. The well was drilled on September 15, 1994, to a depth of 27.5 ft. The water level was at approximately 18 ft below ground surface. Sampling was conducted on April 19, 1997. The well was purged until stabilization of

pH, conductance, dissolved oxygen, and temperature measured using a Hydrolab Monitor (Hydrolab Cooperation, Austin, Texas). At the time of sampling, data were collected by the United States Geological Survey and the Water and Environmental Research Center, University of Alaska Fairbanks. The water table was 20.68 ft below the surface, and at this depth, the water temperature was 5.45 °C and the pH 7.27. The dissolved oxygen was 0.05 mg/l, sulfide 0.009 mg/l, ferrous iron 7.9 mg/l, total iron 8.9 mg/l, hydrogen 0.52 nM, and methane 8.27 ppm. Methods used to measure these variables were discussed in McCarthy et al. (1998). A sterile, disposable Teflon bailer and autoclaved serum bottles were used to collect groundwater samples. The serum bottles were kept in a bag-type glove chamber (Thomas Scientific, Swedesboro, New Jersey) that was purged continuously with nitrogen. The bailer was lowered and lifted slowly in the well to minimize the disturbance of water and to avoid loss of volatile compounds. The bailer was continuously purged with nitrogen. The sterile tip of the bailer was connected to the glove chamber, and groundwater samples were collected in the sterile serum bottles. The serum bottles were closed tightly using butyl rubber stoppers crimped with aluminum seals (Bellco Glass Inc., Vineland, New Jersey). The samples were immediately transported in a cooler to the Water and Environmental Research Center and stored at 4 °C until the following day.

3.2.2 Bacterial Growth Medium

A medium adapted from Edwards and Gribic`-Galic` (1994) was used to support the growth of sulfate-reducing bacteria. Another medium, from Lovely (1986a), was used to support the growth of iron-reducing bacteria. The setup is discussed in the following section. After an acclimation period of one year, modified media were used to support the growth of sulfate-reducing bacteria, iron-reducing bacteria, and methanogenic bacteria in separate incubators. The media prepared to meet the requirements of sulfate reducers, iron reducers, or methanogens is described in Appendix E.

3.2.3 Microcosms and Experimental Design

Experiments were designed to study degradation of benzene by sulfate-reducing bacteria, iron-reducing bacteria, and methanogenic bacteria. Enrichments of microorganisms were prepared the day following sample collection. Two sets of 12 serum bottles were prepared. The first set contained a medium adapted from Edwards and Gribic`-Galic` (1994) that supports the growth of sulfate-reducing bacteria. The second set contained a medium adapted from Lovely (1986a) for the growth of iron-reducing bacteria. All serum bottles and rubber stoppers were autoclaved before they were used. Aquifer samples were transferred to the two sets of 12, 160-ml serum bottles containing medium. Each experimental serum bottle received a 50-ml aquifer sample and 50 ml of the prepared medium.

In each set, four control serum bottles were aseptically prepared, and 50 ml of the medium alone was added to each. All bottles were closed tightly using butyl rubber stoppers and crimped with aluminum seals. Six experimental and two control serum bottles were incubated at room temperature (21 °C) upside down in the dark. Six experimental bottles and two control serum bottles were incubated at 4 °C upside down in the dark. Two μl of benzene (99.9% pure, Aldrich Chemical Company, Milwaukee, Wisconsin) were added to each serum bottle with an acid-washed syringe (800 series syringe multi-pak, Aldrich Chemical Company, Milwaukee, Wisconsin). The active microcosms were fed with approximately 2 μl of benzene (99.9% pure) every month for 10 months as recommended by Edwards and Gribic`-Galic` (1994). After periods of 7 months, 8 months, and 9 months, quantitative measurements were done to confirm the degradation of benzene and to check the production of sulfide and ferrous ions in each set.

After a period of one year, among the active experimental bottles, six microcosms from each group of 12 serum bottles were transferred to a single, autoclaved 1000-ml serum bottle, closed with an acid-washed and sterilized silicon stopper, and sealed with 100% silicon adhesive. The active microcosms were the ones that showed degradation of benzene and production of sulfide and ferrous ions. The control bottles were not

active. This transfer was done in a glove bag purged with N_2/CO_2 (80%/20%) at a temperature of 4 °C.

Three modified media were prepared to support the growth individually, of iron-reducing bacteria, sulfate-reducing bacteria, and methanogenic bacteria. Five hundred ml of each medium were poured into separate, autoclaved 1000-ml serum bottles. The media were autoclaved and cooled while gassing with N_2/CO_2 (80%/20%) in the glove bag. The three bottles were purged with N_2/CO_2 (80%/20%) overnight at a temperature of 4 °C. The following day, 250 ml of the solution from the mixed active microcosms was added to each of the three modified media. The aqueous concentration of benzene was determined using the modified EPA method 602, Federal register (1984): Purge and Trap Capillary Gas Chromatography, HP 7695 Hewlett- Packard Company, Palo Alto, California.

Three sets of 180, 30-ml serum bottles with rubber stoppers were autoclaved. All bottles were filled with 30 ml of one of the three kinds of growth medium, freshly prepared. Microorganisms were transferred from each of the 1000-ml serum bottles prepared earlier, containing 500 ml of medium and 250 ml of solution from active microcosms, to 30-ml serum bottles containing the respective medium. In each set, the serum bottles were divided into 5 equal groups of 36, each containing 12 bottles with each type of medium. One group consisted of the control microcosms:

30 ml of fresh medium, 0.5 mg/250 ml mercuric chloride, and 1 ml of the aqueous culture with 50 parts per billion (ppb) benzene. This group was not autoclaved. The second group consisted of autoclaved control microcosms: 30 ml fresh medium and 1 ml of the aqueous culture with 50 ppb benzene. The samples in these sterile control bottles were autoclaved for 20 minutes at 121 °C. The remaining three groups were test microcosms that consisted of 30 ml fresh medium and 10 ppb benzene, 50 ppb, or 200 ppb and 0.2 ml aqueous culture. The aqueous concentration of benzene was measured and calculated using gas chromatography. All bottles were closed tightly using sterile butyl rubber stoppers and crimped with aluminum seals. Half of each group was incubated at room temperature (21 °C), and the other half at 4 °C in the dark, all upside down. A summary of the experimental setup is given in Appendix F. The bottles were shaken twice daily. Benzene, ferrous and ferric iron, sulfate, sulfide, and protein concentrations were analyzed and the number of bacterial cells were counted monthly, using triplicate samples from every group, over a period of six months (Hutchins et al., 1991, Lovely and Phillips, 1994; Lovely et al., 1994, and Anderson et al., 1998).

After the 6-month period, selected experimental serum bottles were subjected to repeated analyses of benzene, ethylbenzene, p-xylene, and o-xylene concentrations.

In addition, three serum bottles were filled with autoclaved distilled water, and two of these were amended with 35 ppb benzene. One serum bottle amended with benzene was kept at 4 °C, and the other two were kept at room temperature (21 °C). These serum bottles were subjected to BTEX analysis over a period of 190 days. Another setup was prepared to provide iron-reducing conditions and the loss of benzene was observed over 35 days. Further details of this aspect of the experiment will be discussed in the following chapters.

All preparations were done in a bag-type portable glove chamber that had been purged overnight with N_2/CO_2 (80%/20%) in a cold room at 4 °C, approximately the ambient temperature of groundwater. Any traces of oxygen in the purge gas mixture were removed by passing the gas mixture through an Oxiclear Disposable Purifier (Alltech, Deerfield, Illinois).

3.2.4 BTEX Analysis

EPA method 602- Purge and Trap Capillary Gas Chromatography (5890 Hewlett- Packard Company, Palo Alto, California; Federal Register, 1984) was used to analyze for BTEX in all the samples. To satisfy the requirements, a HP 7695 purge and trap concentrator was set up in series with an automatic sampler and HP series II gas chromatograph. However, because a photoionization detector (PID) was not available, a flame ionization detector (FID) was utilized in its place. To maximize the

separation capability of the system, an HP-1 column and a Restek RTX-1 column were connected in series. The HP-1 was coated with non-polar bonded phase (100%-polydimethylsiloxane). The length of the column was 30 meters. and it had an inside diameter of 0.53 mm. The film thickness was 1.5 µm. The RTX-1 was a 30 meter megabore column coated with cross-bonded polydimethylsiloxane stationary phase, compatible with the HP-1 column. It had a 0.53-mm inside diameter and a film thickness of 0.25 µm.

A 5-ml gas-tight syringe (Hamilton Co., Reno, Nevada) was used to transfer samples from the serum bottles to the purge and trap device. For each sample, 5 ml of liquid sample was injected into the reactor of the purge and trap auto-sampler. Nitrogen gas was then purged through the reactor for 12 minutes to effectively transfer all volatile compounds to a trap. The trap was a 25-cm column packed with Tenax. The trap retained BTEX until the desorb cycle was initiated. During this cycle, the trap was heated to 18 °C. The heat caused the trap to release all volatile compounds into the nitrogen stream, which entered the gas chromatograph.

The aqueous concentration of aromatic hydrocarbons was determined by comparing peak areas in samples with those of external certified standards (Rescek Corporation, Bellefonte, Pennsylvania). BTEX standards were prepared by diluting more concentrated standards and

analyzing along with samples. At least 10 standards were prepared for each calibration curve, ranging from 0 to 200 μg/l. The relationship between the chromatographic peak area and the concentration of the standards was determined by performing regression analysis (See Appendix G). The concentration of aromatic compounds in the headspace was calculated using Henry's Law constants at 20 °C and 4 °C obtained from the literature (Mackay and Shiu, 1981; Bamford et al., 1998).

3.2.5 Extraction and Gas Chromatography/Mass Spectrometry (GC/MS)

3.2.5.1 Extraction and Concentration of Potential Intermediates

During the experiment, a 50-ml sample from the test serum bottles and 50 ml of methylene chloride were added to a 250-ml separatory funnel. The funnel was shaken vigorously for 3 minutes. The 2 liquids were allowed to separate for 10 minutes. Afterwards, the solvent phase was transferred to a Kuderna-Danish (KD) apparatus. The KD apparatus was then immersed in a water bath at 53 °C, which caused the methylene chloride to vaporize. At the end of the concentration process, the concentrated sample was diluted with methylene chloride to 1 ml and dried with anhydrous sodium sulfate.

3.2.5.2 GC/MS Analysis of Potential Intermediates

Analysis was done using an HP 6890 gas chromatograph interfaced with a HP 5973 mass spectrometer. The GC was equipped

with a HP-5 MS capillary column. The microbore column was 30 m long, with a diameter of 0.25 μm film of 5%-diphenyl-95% dimethylsiloxane copolymer. The GC/MS temperature parameters are listed in Table 3.1:

Table 3.1: GC/MS temperature parameters for the determination of potential intermediates

Injection Temperature = 280 °C
Initial Temperature = 45 °C
Final Temperature = 300 °C
Interface Temperature = 280 °C
Program Temperature= 250 °C

3.2.6 Cell Count, Morphology, and Preservation of Bacteria

3.2.6.1 Total Number of Bacteria

The total number of bacteria (TNB) was determined by using an acridine orange (0.01%) staining technique. Acridine orange was prepared by dissolving 10 mg acridine orange in 1 liter of 6.6 mM sodium potassium phosphate buffer, pH 6.7. The phosphate buffer was a mixture of 2.45 mM KH_2PO_4 and 4.15 mM Na_2HPO_4 in deionized water (Bratbak, 1993). All solutions were autoclaved and filter-sterilized (0.1μm pore size). Once a month for a period of six months, 10 μl samples were collected from all the serum bottles using a sterile syringe. The samples were spread over 1 cm^2 on clean microscope slides. The slides were heat

fixed and stained with acridine orange for 2 minutes and then rinsed with tap water and air dried (Edwards and Gribic`-Galic`, 1994). The cells were observed under oil immersion using an epifluorescence microscope equipped with a x100 objective lens and x10 ocular lens (Zeiss-West Germany); 10-15 fields were counted per sample. The average cell count was determined from the count field and the area of each field was 0.01 mm^2. Photographs were taken via an attached camera (MC 63 photomicrographic camera with 35-mm film) (See Chapters 4 and 5).

3.2.6.2 Modified Hucker Gram Staining Method

The modified Hucker Gram Staining Method (Gerhardt et al., 1994) was also used. Gram stain (Defco Laboratories, Detroit, Michigan) was applied to selected samples to determine if the bacteria were Gram positive or Gram negative. These samples were collected during the first, third and sixth months. One bottle from each set at each concentration for the three types of cultures was selected. Samples were spread on clean microscopic slides. The smears were heat fixed by passing the slides over a low flame. The slides were cooled and flooded with Gram Crystal Violet for 1 minute and washed with cold tap water. The slides were then flooded with mordant Bacto gram iodine for 1 minute. The mordant was removed by washing the slide with tap water. Bacto Gram Decolorizer was poured over the slide until the slides became colorless, for around 30-60 seconds, and then the slides were washed with tap water, flooded with

counterstain, Bacto Gram Safranin, for 30-60 seconds, and washed with cold tap water. The slides were allowed to air dry and subsequently examined under the oil immersion lens.

3.2.6.3 Schaeffer-Fulton Endospore Test

The Schaeffer-Fulton staining method (Gerhardt et al., 1994) was applied to determine the presence of endospores. Selected cultures were exposed to –20 °C in a cold room or to 80 °C in a dry oven for a period of one hour. These samples were collected after the fifth month of incubation. The selection included one bottle from each set, at 50 ppb and 200 ppb benzene concentration, for each of the three types of cultures. Smears from cultures exposed and not exposed to extreme temperature were prepared, air dried, and heat fixed on clean microscope slides. The slides were covered with 5% aqueous malachite green for 30-60 seconds and heated to steaming 3 or 4 times. After washing the excess stain from the slides with tap water, the slides were stained with 0.5% aqueous safranin for 30 seconds. Then the slides were washed in running water and blotted dry. The slides were examined under high-dry and oil-immersion lens systems. Spores appeared light green and the rest of the cells brownish-red under the lighted microscope.

3.2.6.4 Dugid Staining Method

The Dugid staining method (Gerhardt et al., 1994) was used to check for the presence of capsules that are produced by some bacteria

under specific culture conditions. Samples were collected after the fifth and sixth months of incubation. The selection included one bottle from each set at each benzene concentration for the three types of cultures. A large loopful of India ink was placed on a clean microscope slide and mixed with a loopful of culture. Part of the mixture was covered with a glass cover slip, which was pressed down firmly. The slide was examined under high-dry and oil-immersion lens systems. Capsules appeared as clear areas around the organisms, with a brownish black background full of dancing particles of India ink.

3.2.6.5 Hiss Staining Method

The Hiss staining method (Gerhardt et al., 1994) was also used to check for the presence of capsules that are produced by some bacteria under specific culture conditions. Samples were collected after the fifth and sixth months of incubation. The selection included one bottle from each set at each benzene concentration for the three types of cultures. A loopful of culture was mixed with a drop of skim milk on a clean microscope slide, air dried, and heat fixed. The slide was covered with crystal violet stain (0.1 g crystal violet/ 100ml distilled water) and heated until steam rose. Then the slide was washed with a 20% (wt/vol) aqueous solution of copper sulfate and blotted dry. The slide was examined under an oil-immersion lens system. Capsules appeared faint blue and organisms appeared dark purple.

3.2.6.6 Preservation of Bacteria on Filter Paper

This method was simple and inexpensive. Bacteria were preserved by drying a suspension on sterile filter paper strips and storing them (Gerhardt et al., 1994). All preparation was done in a bag-type portable glove chamber that had been purged with N_2/CO_2 (80%/20%) in a cold room at 4 °C. Sterile filter paper strips were placed in sterile petri plates. The bacterial suspension was poured over the paper strips until the strips became saturated. Then the strips were removed using sterile forceps and placed into different sterile petri dishes where they were allowed to dry. The dry filter paper strips were stored in anaerobic tubes, purged with N_2/CO_2 (80%/20%), closed with rubber stoppers, and crimped with aluminum seals in a cold room at the ambient temperature of groundwater (4 °C). By this method, it was expected that bacteria would be preserved for about two years.

3.2.6.7 Preservation of Bacteria by Deep Freezing

Cultures were transferred to centrifuge bottles in an anaerobic glove bag purged with N_2/CO_2 (80%/20%). The bottles were tightly closed and centrifuged (5000 rpm, 45 minutes) (Sorvall RC-SC and SB Plus Du Pont, Wilmington, Delaware). The suspension was removed and the pellets were resuspended in 1 ml of fresh medium in Eppendorf tubes. Seventy μl dimethylsulfoxide (DMSO), a cryoprotective agent, was added

to each tube (Edwards and Grabic`-Galic`, 1994). The tubes were mixed and flash-frozen in liquid nitrogen. Immediately, the frozen cells were stored at - 80 °C in a dry freezer at Dr. Gerald Plumley's laboratory, Institute of Marine Science, University of Alaska Fairbanks.

The centrifuge bottles used in the previous method were not gas-tight. Another method was used to deep freeze bacteria in an anaerobic glove bag purged with N_2/CO_2 (80%/20%). A 1.5 ml sample of the cultures was transferred to safe-seal microcentrifuge tubes (Sorenson, Salt Lake, Utah). The tubes were centrifuged (5000 rpm, 10 minutes) in a Biofuge 13 centrifuge (Baxter Scientific products, Phillipsberg, New Jersey). The suspension was removed, and the pellets were resuspended with 1 ml of fresh medium in Eppendorf tubes. Seventy μl dimethylsufoxide (DMSO), a cryoprotective agent, was added to each tube. The tubes were mixed and flash-frozen in liquid nitrogen. Immediately, the frozen cells were stored at – 80 °C.

3.2.7 Sulfate Analysis

The methods in this section were only applied to the culture that supported the growth of sulfate-reducing bacteria. Sulfate was determined qualitatively and quantitatively using analytical test strips (EM Science, Gibbstown, New Jersey) for the detection of sulfate ions. If the concentration of sulfate was below 1600 mg/l, then the sulfate concentration was measured quantitatively using the Standard Method

4500-SO_4^{2-} : Gravimetric method with Drying Residue (Greenberg et al., 1992). The method involved precipitating the sulfate with barium chloride. Five ml of the sample was added to 150 ml of deionized water. The pH was adjusted to 5 by adding 1 ml HCl . The mixture was heated to boiling. A barium chloride solution was added slowly until precipitation was complete. The mixture was kept overnight in a 90 °C oven. The resulting barium sulfate precipitate was then filtered onto a membrane filter with a pore size of 0.45 μm (Costar Scientific Corporation, Cambridge, Massachusetts). The weight of the membrane filter before and after collection of the barium sulfate precipitate was measured and recorded. The filter and precipitate were dried overnight at 105 °C and the weight was recorded again.

3.2.8 Modified Standard Method 4500-S^- -Methylene Blue

The method in this section was only applied to the culture that supported the growth of sulfate-reducing bacteria. Sulfide concentration was determined by using Modified Standard Method 4500-S^- : Methylene Blue Residue (Greenberg et al., 1992). This method was based on the reaction of sulfide, ferric chloride, and dimethyl-*p*-phenylenediamine to produce methylene blue. Ammonium phosphate was added after color development to remove the ferric chloride color.

Reagents used in this analysis were prepared and stored at room temperature in amber glass bottles wrapped with aluminum foil. The

reagents were amine-sulfuric acid stock solution, amine-sulfuric acid reagent, ferric chloride solution, sulfuric acid solution, and diammonium hydrogen phosphate solution, and methylene blue solution.

The amine-sulfuric acid stock solution consisted of 2.7 g N,N-dimethyl-p-phenylenediamine oxalate dissolved in 5 ml concentrated H_2SO_4 and 2 ml distilled water. This mixture was diluted in 10 ml distilled water after it cooled. This stock was prepared fresh for all analyses because an old supply might be oxidized and discolored in a way that would interfere with the colors in this test.

The amine sulfuric acid reagent consisted of 2.5 ml of amine-sulfuric acid stock solution diluted in 97.5 ml 9M H_2SO_4. This stock was prepared fresh prior to each analysis. The ferric chloride solution consisted of 100 g $FeCl_3.6H_2O$ in 40 ml water. The diammonium hydrogen phosphate solution consisted of 200 g $(NH_4)_2HPO_4$ dissolved in 400 ml distilled water.

The methylene blue solution I consisted of one gram of USP methylene blue dye dissolved in 1 L of distilled water. It was standardized against sulfide, so that 0.05 ml (one drop) = 1.0 mg sulfide/L. For methylene blue solution II, 10 ml of the adjusted methylene blue solution I was diluted in 100 ml distilled water.

Several grams of clean crystals of $Na_2S.9H_2O$ were dissolved in enough water to cover the crystals. A varying number of drops of this

solution were added to a series of volumetric flasks each containing 1 liter of deionized water. Samples (7.5 ml) of the prepared solutions were transferred to each of 2 test tubes, A and B. Amine sulfuric acid reagent (0.5 ml) and 3 drops of ferric chloride solution were added to tube A and mixed immediately. Sulfuric acid solution (0.5 ml) and 3 drops of ferric chloride reagent were added to tube B. The presence of S^{-2} was indicated by the appearance of blue color in tube A. The color development was complete in around 3 minutes. Diammonium hydrogen phosphate solution (1.6 ml) was added to each solution. After 15 minutes, a color comparison was done by adding methylene blue solution I and/or II to tube B until a color match developed with the first tube. The concentration of the standards was estimated by visual color comparison, by the number of drops of methylene blue added. The sulfide concentration was calculated as: mg S^{-2}/L = no. of drops solution I + 0.1 (no. of drops solution II).

Color was measured for both tubes A and B using a spectrophotometer (Spectronic 2000, Bausch and Lomb) at a wavelength of 675 nm. The wavelength was determined after measuring different concentrations of the standards to determine the peak absorbance. A correction was made depending on the difference in absorbance between tube A and tube B. A calibration curve plotting the absorbance vs. the concentration of the standards was used to determine the concentration of

sulfide in unknown samples (See Appendix G). This method can detect sulfide concentrations above 0.1 mg/L. The samples were diluted if the reading exceeded 20 mg/L, which was the upper limit of this method.

3.2.9 Modified Standard Method-Fe Phenanthroline

The method in this section was only applied to the culture that supported the growth of iron-reducing bacteria. It was used to measure ferrous and total iron concentration. Ferric concentration was calculated by subtracting the ferrous concentration from total iron concentration. Reagents used in this analysis were prepared and stored in dark glass bottles. The pH of iron standard stock solutions was adjusted to 1.5 to 2 by adding HCl. In order to measure the unknown concentration of ferrous iron in the samples, fresh ferrous standards were prepared in the range of 0 -3000 ppb Fe. Twenty-five ml of each standard and 3.5 ml of Fe^{+2} were added to 50 ml volumetric flasks. Ten ml phenanthroline solution and 5 ml ammonium acetate buffer were added. Distilled water was added to the volumetric flask to bring the volume to 50 ml. The same steps were followed, with the addition of 0.5 ml hydroxylamine, in order to measure the total iron concentration. Color measurement was done using the spectrophotometer at a wavelength of 510 nm. A calibration curve plotting the absorbance vs. the concentration of the standards was used to determine the concentration of the ferrous and total iron in the unknown samples (Appendix G).

Reagents used in this analysis were prepared and stored at room temperature in volumetric flasks wrapped with aluminum foil. The reagents were stock (Fe $(NH_4)_2(SO_4)_2$-$6H_2O$), hydroxylamine solution, ammonium acetate buffer, and phenanthroline. All reagents were stored at 4 °C except the buffer which was kept at room temperature.

The stock Fe solution consisted of 1.404 g ($Fe(NH_4)_2(SO_4)_2$-$6H_2O$ per liter of deionized water. The hydroxylamine solution was prepared by dissolving 10 g $NH_2OH.HCl$ in 100 ml H_2O. The ammonium acetate buffer consisted of 250 g $NH_4C_2H_3O_2$ in 150 ml H_2O and 700 ml concentrated glacial acetic acid. The phenanthroline solution was prepared by dissolving 1 g (1,10) phenanthroline, and 1 ml HCl per liter of deionized water.

3.2.10 Sigma Procedure No. TPRO-562

This method is based on the bicinchoninic acid (BCA) detection reagent proposed by Smith et al. (1985). BCA is a water-soluble compound and a chromogenic reagent for cuprous ion, Cu (I), forming a purple complex. Proteins reduce alkaline Cu (II) to Cu (I). The addition of BCA to protein and Cu (II) results in a color production that is directly proportional to the protein concentration. For my samples, the concentration of the unknowns was determined from a plot of absorbance vs. concentration obtained for the standard protein solution. A standard curve was prepared by plotting the reported absorbance vs. the known

protein concentration (Appendix G). Two ml protein determination reagent (1 part copper (II) sulfate pentahydrate 4% solution to 50 parts bicinchoninic acid solution) was added to sets of tubes containing the protein standards and 0.1 ml of unknown samples. The tubes were vortex mixed and incubated at 37 °C for 30 minutes, then cooled at room temperature. The absorbance of the standards and the unknown samples was determined at 562 nm using a spectrophotometer. Deionized water was used as the reference. Some samples required dilution, if the net absorbance at 562 nm fell outside the range of the standard curve. The absorbance of the blank was subtracted from that of standards and samples to obtain the net absorbance due to protein. The standard curve was used to determine the amount of protein in the unknown samples. The protein concentration was calculated by multiplying the unknown protein per assay (mg) by the dilution factor and dividing by the volume of the unknown used for the assay (ml) (Sigma procedure No. TPRO-562):

$$P_c = \frac{P \times D}{S}$$

Where:

P_c = protein concentration in mg/ml
P = unknown protein per assay in mg
D = dilution factor
S = unknown sample used for assay in ml

CHAPTER FOUR

EXPERIMENTS TO ENRICH FOR BENZENE DEGRADATION BY SULFATE- AND IRON- REDUCING BACTERIA

4.1 Introduction

The soil and groundwater in Operable Unit 5 (OU5) at Fort Wainwright, Alaska, have become contaminated with petroleum hydrocarbons as a result of leakage from underground tanks, accidental spills, and improper disposal techniques. Such groundwater contamination is a widespread problem in the United States (U.S. Environmental Protection Agency, 1994). However, OU5 groundwaters are unusual because of their very low temperature, 4 °C. The most water-soluble components of petroleum contaminants are the homocyclic aromatic compounds, benzene, toluene, ethylbenzene, and xylene (BTEX). The indigenous microorganisms in BTEX-contaminated groundwater can use the contaminants as a source of carbon, energy, and building blocks for new bacterial cells. When aerobic bacteria use the contaminants in this way, they use dissolved oxygen as a terminal electron acceptor. The consumption of oxygen may result in anaerobic conditions if the rate exceeds the rate of groundwater, and some groundwaters are anoxic even in the absence of contaminants, due to high biological oxygen demand or slow recharge. Research is needed to study the biodegradation of benzene and other hydrocarbons under anaerobic and low temperature conditions to better evaluate the potential

contribution of anaerobic bioremediation to contaminated site restoration in high-latitude regions.

Most past attempts to show that benzene can be biodegraded in an anaerobic environment failed under methanogenic conditions (Schink 1985; Wilson and Kampbell, 1992). However, the research of Edwards and Gribic`-Galic` (1992, 1994), Edwards et al. (1992), Evans et al. (1991), and Haag et al. (1991) indicated that bioremediation of groundwater contaminated with aromatic hydrocarbons, including benzene, is possible using mesophilic anaerobic bacteria. Vogel and Gribic`-Galic` (1986) showed that the anaerobic oxidation of toluene and benzene was initiated by oxygen derived from ^{18}O-labeled water, and that both toluene and benzene were fermentatively oxidized with ^{18}O from ^{18}O-labeled water. A variety of alternate electron acceptors such as nitrate (Kuhn et al., 1987; Reinhard et al., 1997), ferric iron (Lovely and Lonergan, 1990; Anderson et al., 1998), and sulfate (Lovely et al., 1995; Reinhard et al., 1997) also support microbial degradation of aromatic pollutants.

Zobell and Agosti (1972), and Kerry (1990) found that psychrophilic microorganisms have the ability to degrade a variety of contaminants aerobically. However, anaerobic biodegradation of aromatic hydrocarbons at temperatures below 10 $^{\circ}C$ in the presence of electron acceptors such as SO_4^{-2} and Fe^{+3} has not been studied in detail previously.

Sulfate-reducing bacteria belong to the kingdom Eubacteria and are active in strictly anaerobic environments. Sulfate-reducing bacteria cannot degrade natural polymers, so they depend on fermentation products of other anaerobic bacteria for carbon and energy sources. They can outcompete methanogens and acetogens for such common substrates as H_2 and acetate (Oremland and Taylor, 1978; Horikoshi and Grant, 1991). Oremland and Marsh (1982) also showed that sulfate reduction and methanogenesis could occur concurrently, if sulfate reducers and methanogens utilize different electron donors or one group provides an electron donor needed by the other group.

Iron-reducing microorganisms can outcompete sulfate reducers and methanogens for hydrogen and acetate (Lovely and Phillips, 1987a). If iron is being reduced, the hydrogen partial pressure is lower than under conditions where methanogenic and sulfate-reducing bacteria predominate. Also the iron-reducing bacteria maintain a low concentration of H_2 and acetate in a way that inhibits the production of sulfate-reducing bacteria and methanogenesis (Lovely, 1986a, b). Although the complete oxidation of fermentable substrates to CO_2 and the transfer of all the electrons to Fe (III) is thermodynamically favorable, only a minor portion of the electrons from fermentable substrates are transferred. Yet, iron-reducing bacteria are not excluded from metabolizing fermentable

products and mineralizing organic matter with ferric iron as the sole electron acceptor (Lovely, 1986a, b). The end product is ferrous iron.

The primary goal of this research was to obtain information about the biodegradation of benzene and other BTEX compounds in soil and groundwater, under iron- and sulfate-reducing conditions at low temperature. Understanding the transformation of benzene and other compounds could help to predict the fate of contamination in soil and groundwater in cold regions. More specifically, the purpose of the work presented in this paper was: (1) to determine the degree of transformation and mineralization of benzene under anaerobic conditions, at temperatures of 4 ^{o}C and 21 ^{o}C; (2) to identify the role of the microorganisms in the mineralization of the BTEX compounds; (3) to determine the response of the microorganisms to variations of environmental factors including temperature and substrate concentration; and (4) to partly characterize the microbial consortium involved in the biodegradation of benzene and other hydrocarbons.

4.2 Materials and Methods

(See sections 3.2.1-3.2.10)

4.3 Results

The benzene data presented in this chapter are based on the mean of three replicates. The benzene data for each serum bottle analyzed every month over a period of six months are presented in Appendix H.

These data usually include three benzene concentrations, and the mean, standard deviation and the 90% confidence intervals calculated for a small population based on the small sampling theory (Spiegel, 1994). The confidence coefficient (t_c) value is selected based on the confidence desired; in this case the value used was 90%.

Each replicate serum bottle developed independently over the 6 month incubation period. Hence some differences among replicate bottles were substantially greater than that expected due to analytical error. However, benzene concentrations of replicates were generally in close agreement; the mean standard duration of control replicates at 50 ppp was 6.5 ppb and the relative standard deviation averaged 11 %. For the experimental active bottles nearly all concentrations measured were less than 10 ppb. For the experimental active bottles the standard deviation for samples ≤ 10 ppb averaged 2 ppb. Larger differences among replicate bottles were observed at one month in some of the biologically active bottles. In these cases, iron-reducing conditions at 50 and 200 ppb benzene and at 4 and 21 °C, some of the replicate bottles had decreased to very low concentrations, while benzene concentration in some replicates remained higher. This indicates that there was considerable variability in the benzene degradation rate initially, but by two months benzene concentrations were ≤ 12 ppb in almost all biologically active replicates under all conditions.

The pH was 7 and remained constant during the period of incubation. Anaerobic conditions throughout the incubation period were confirmed by the clear color of the redox indicator, resazurin, used in the experiment.

4.3.1 Benzene Degradation

4.3.1.1 Sulfate Reduction Experiments

Figures 4.1-4.4 summarize the degradation of benzene under sulfate-reducing conditions. The initial benzene concentrations in the experimental samples were 10, 50, and 200 ppb and in the controls, 50 ppb. Decrease in benzene, except controls, within a period of six months is shown as the mean of three replicates in Table 4.1 and Figure 4.5. Loss of benzene within 6 months at 21 °C was 57 % at 10 ppb, 100% at 50 ppb benzene, and approximately 93 % at 200 ppb. Disappearance of benzene within six months at 4 °C was 100% at 10 and 50 ppb benzene, and approximately 98 % at 200 ppb.

The concentration of benzene in the headspace, calculated by using Henry's law (Mackay and Shiu, 1981; Bamford et al., 1998), is shown in Appendix I. The headspace concentration was ≤ 20% of the total at 21 °C and ≤ 10% of the total at 4 °C. At the end of the six month period, there was no significant loss to volatization or other non-biological processes.

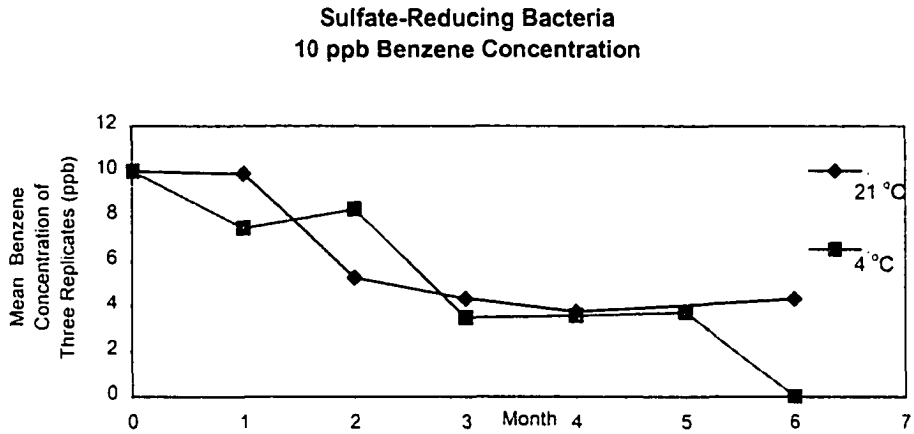

Figure 4.1: Biodegradation of 10 ppb of benzene under sulfate-reducing conditions over a period of six months at 21 °C and at 4 °C. The initial benzene concentration was calculated.

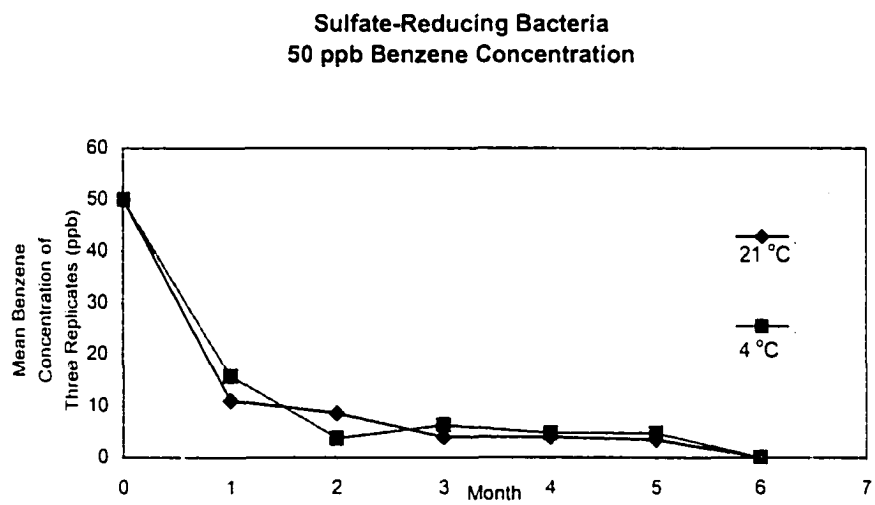

Figure 4.2: Biodegradation of 50 ppb of benzene under sulfate-reducing conditions over a period of six months at 21 °C and at 4 °C. The initial benzene concentration was calculated.

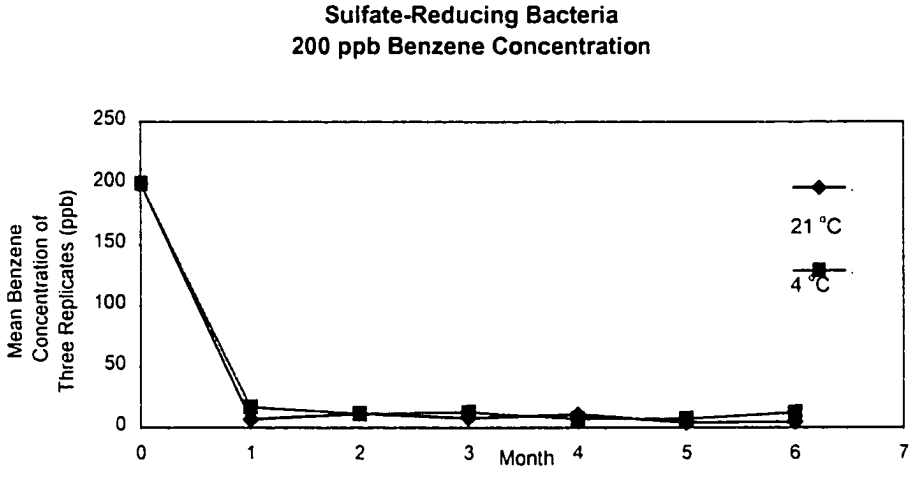

Figure 4.3: Biodegradation of 200 ppb of benzene under sulfate-reducing conditions over a period of six months at 21 °C and at 4 °C. The initial benzene concentration was calculated.

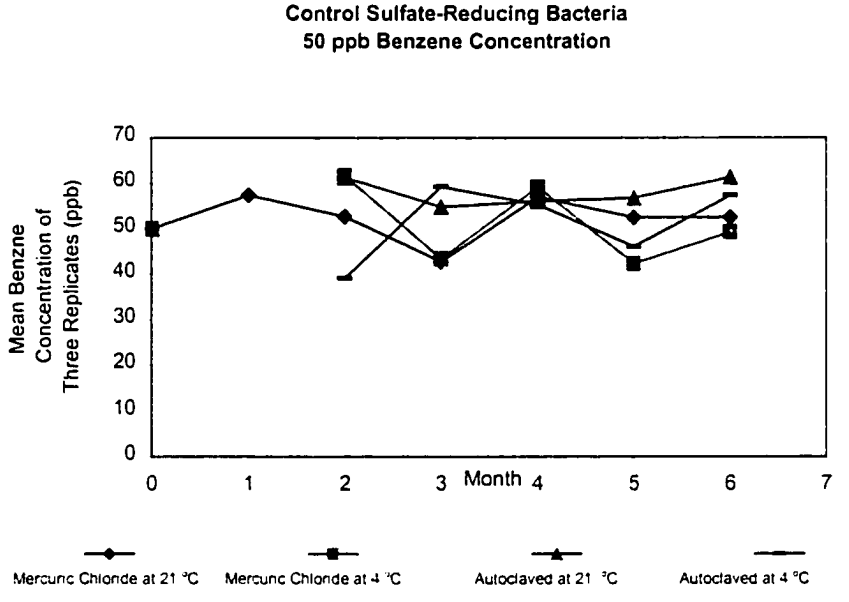

Figure 4.4: Observation of 50 ppb of benzene in the control serum bottles under sulfate-reducing conditions over a period of six months at 21 °C and at 4 °C. The initial benzene concentration was calculated.

During the first two months, the concentration of benzene dropped rapidly, at least at the 50 and 200 ppb concentrations, but the apparent rate of decrease slowed after the second month. Initially, the decrease was faster at 21 °C than 4 °C when the benzene concentration was 200 ppb (Figures 4.2-4.3), but there was no temperature difference at the other two concentrations. After a period of two months, the apparent benzene degradation rate was about the same at 21 °C and 4 °C (Figures 4.2-4.3) at all concentrations. Complete removal of benzene occurred at 21 °C and 4 °C for serum bottles amended with 50 ppb benzene (Figure 4.2), but not for those amended with 200 ppb benzene (Figure 4.3). (See Appendix H). When the benzene concentration was 10 ppb, benzene removal was complete at 4 °C but not at 21 °C (Figure 4.1). Neither the chemically killed nor the autoclaved controls showed significant losses of benzene at either 4 °C or 21 °C.

Apparently, ferrous iron added to the medium precipitated with sulfide ions as ferrous sulfide. Black precipitate, assumed to be ferrous sulfide (Beller et al., 1992; Gupta et al., 1994b) in the culture medium was observed in the serum bottles that were amended with 200 ppb of benzene at 21 °C. There was no black precipitate or detection or odor of hydrogen sulfide at 4 °C, except after six months in the bottles that were amended with 200 ppb benzene.

Table 4.1: Cumulative degradation (%) over a period of six months under conditions favorable to sulfate-reducing bacteria. The concentrations of benzene are the mean of the three replicates measured every month. The month-0 concentration was calculated.

Sulfate-Reducing Bacteria			Sulfate-Reducing Bacteria		
Benzene 10 ppb at 21 °C			**Benzene 50 ppb at 4 °C**		
Month	Benzene (ppb)	Σ Degradation %	Month	Benzene (ppb)	Σ Degradation %
0	10	0	0	10	0
1	12.0	0	1	7.5	25
2	5.3	47	2	8.3	17
3	4.4	56	3	3.5	65
4	3.8	62	4	3.6	64
6	4.3	57	5	3.4	100
			6	0	100
Sulfate-Reducing Bacteria			**Sulfate-Reducing Bacteria**		
Benzene 50 ppb at 21 °C			**Benzene 50 ppb at 4 °C**		
Month	Benzene (ppb)	Σ Degradation %	Month	Benzene (ppb)	Σ Degradation %
0	10	0	0	50	0
1	11.0	78	1	15.8	68
2	8.7	83	2	3.7	92
3	4.0	93	3	6.3	87
4	3.5	93	4	4.7	91
5	3.5	100	5	2.7	96
6	0	100	6	0	100
Sulfate-Reducing Bacteria			**Sulfate-Reducing Bacteria**		
Benzene 200 ppb at 21 °C			**Benzene 200 ppb at 4 °C**		
Month	Benzene (ppb)	Σ Degradation %	Month	Benzene (ppb)	Σ Degradation %
0	200	0	0	200	0
1	7.1	96	1	17	92
2	11.7	94	2	11.8	94
3	8.1	96	3	12.6	94
4	10.8	95	4	7.2	96
5	4.1	98	5	5.7	97
6	4.6	98	6	12.5	94

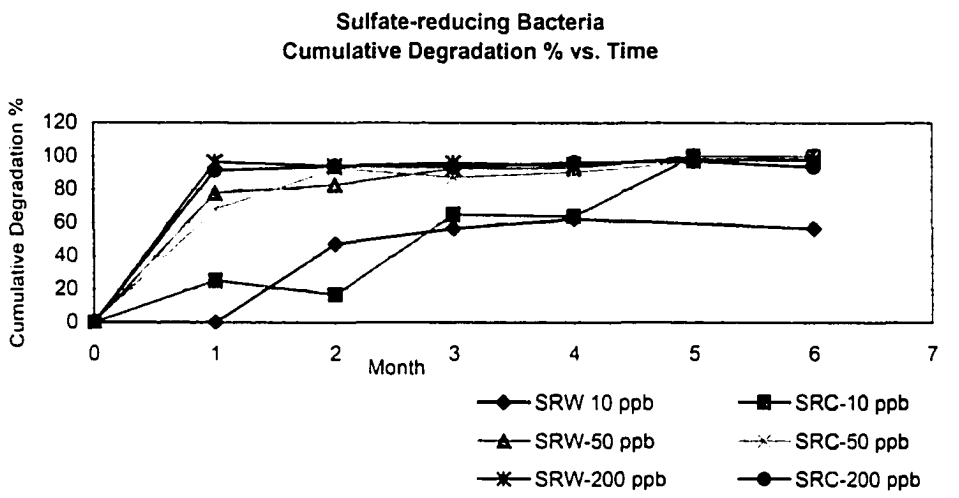

Figure 4.5: Cumulative degradation (%) over a period of six months under conditions favorable to sulfate-reducing bacteria. The percentage of benzene are the mean of the three replicates measured every month.
SRW-10 ppb = Sulfate-reducing conditions, amended with 10 ppb benzene at 21 °C
SRC-10 ppb = Sulfate-reducing conditions, amended with 10 ppb benzene at 4 °C
SRW-50 ppb = Sulfate-reducing conditions, amended with 50 ppb benzene at 21 °C
SRC-50 ppb = Sulfate-reducing conditions, amended with 50 ppb benzene at 4 °C
SRW-200 ppb = Sulfate-reducing conditions, amended with 200 ppb benzene at 21 °C
SRC-200 ppb = Sulfate-reducing conditions, amended with 200 ppb benzene at 4 °C

4.3.1.2 Iron Reduction Experiments

In a parallel experiment, iron was the electron acceptor and benzene served as the substrate. Figures 4.6-4.9 show the concentration of benzene under iron-reducing conditions. The initial concentrations in the experimental samples were 10, 50, and 200 ppb and in the controls, 50 ppb.

Benzene degradation over a period of six months is summarized as the mean of three replicates in Table 4.2 and Figure 4.10.
The concentration of benzene in the headspace of each bottle, calculated by using Henry's law (Mackay and Shiu, 1981: Bamford et al., 1998), is summarized in Appendix I. At the end of the six month period, there was no significant loss to volatization or other non-biological processes.

Benzene loss was 100 % within 6 months at 10 and 50 ppb benzene, and approximately 98 % at 200 ppb, at both 21 °C and 4 °C. During the first two months, the concentration of benzene dropped rapidly, but the decrease in concentration slowed after the second month, when the remaining benzene concentration was less than 13 ppb. Initially, the rate appeared faster at 21 °C than 4 °C, in some of the bottles with initial benzene concentrations of 50 and 200 ppb (Figures 4.6-4.8). After two months (when the concentration of benzene was low) the rate of biodegradation of benzene was slower at 21 °C than 4 °C, for initial

benzene concentrations of 50 ppb and 200 ppb (Figure 4.7), but temperature had no effect at the other two initial concentrations. Neither the chemically killed nor the autoclaved controls showed any degradation of benzene.

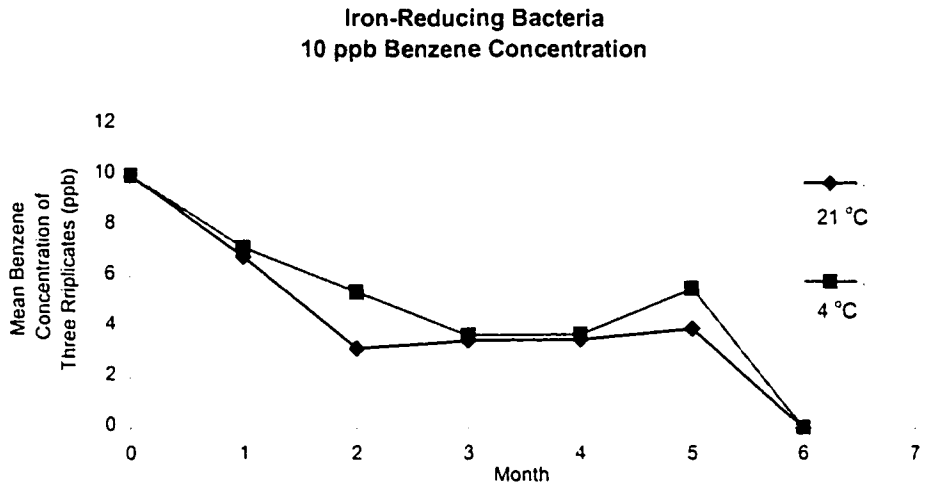

Figure 4.6: Biodegradation of 10 ppb of benzene under iron-reducing conditions over a period of six months at 21 °C and at 4 °C. The initial benzene concentration was calculated.

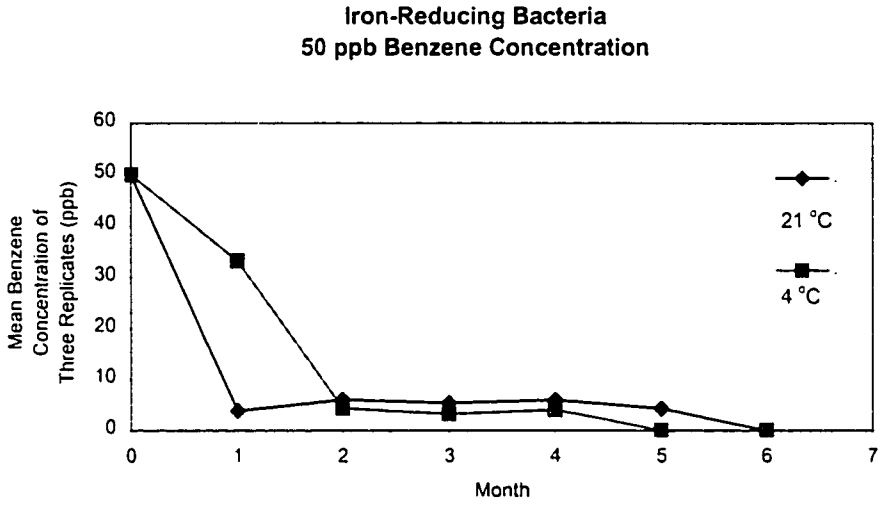

Figure 4.7: Degradation of 50 ppb of benzene under iron-reducing conditions over a period of six months at 21 °C and at 4 °C. The initial benzene concentration was calculated.

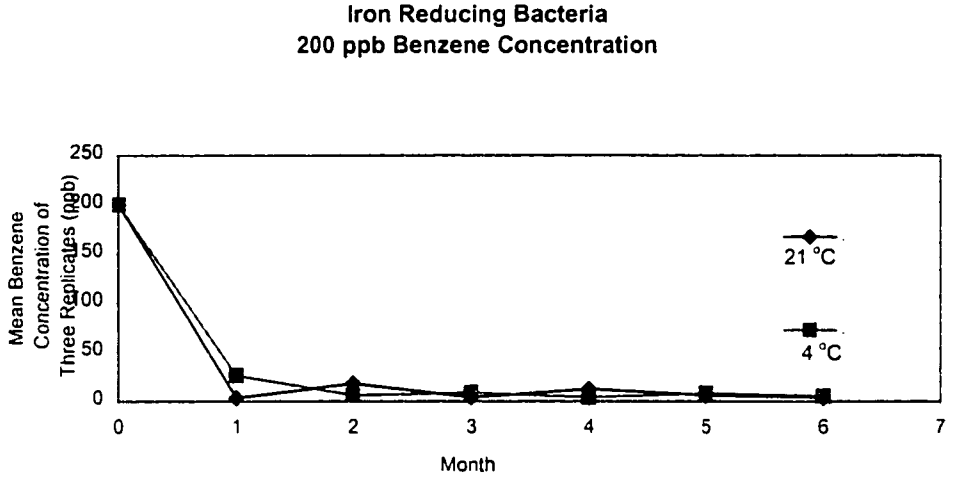

Figure 4.8: Biodegradation of 200 ppb of benzene under iron-reducing conditions over a period of six months at 21 °C and at 4 °C. The initial benzene concentration was calculated.

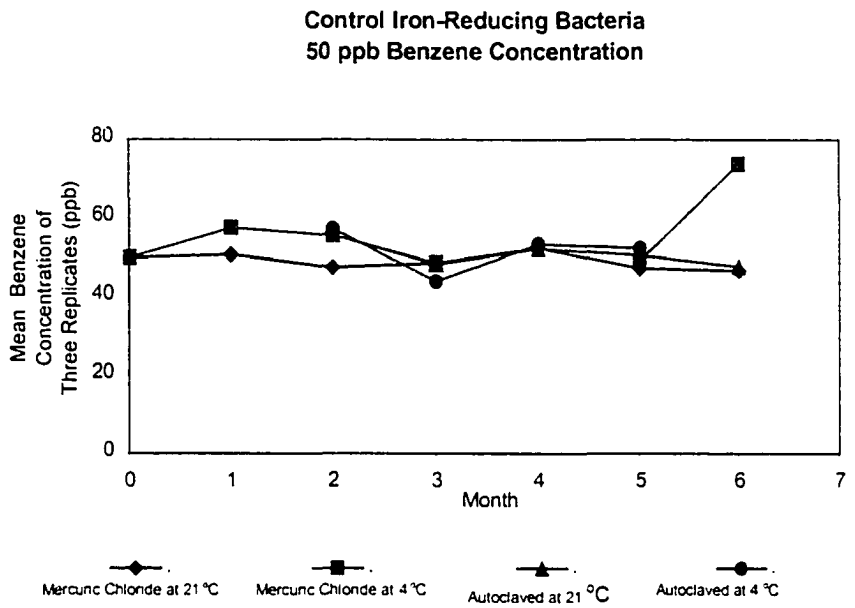

Figure 4.9: Biodegradation of 50 ppb of benzene in the control serum bottles under iron-reducing conditions over a period of six months at 21 °C and at 4 °C. The initial benzene concentration was calculated.

As in some of the sulfate reduction bottles, sulfide added to the medium apparently precipitated with ferrous ions as ferric sulfide. The black precipitate was greater at higher initial benzene. The occurrence of the black precipitate seemed to occur earlier at 21 °C than at 4 °C.

Table 4.2: Cumulative degradation (%) over a period of six months under conditions favorable to iron-reducing bacteria. The concentrations of benzene are the mean of the three replicates measured every month.

Iron-Reducing Bacteria			Iron-Reducing Bacteria		
Benzene 10 ppb at 21 °C			**Benzene 10 ppb at 4 °C**		
Month	Benzene (ppb)	Σ Degradation %	Month	Benzene (ppb)	Σ Degradation %
0	10	0	0	10	0
1	6.8	32	1	7.1	29
2	3.2	68	2	5.2	46
3	3.5	65	3	3.7	63
4	3.5	65	4	3.7	63
5	3.9	61	5	5.5	45
6	0	100	6	0	100
Iron-Reducing Bacteria			**Iron-Reducing Bacteria**		
Benzene 50 ppb at 21 °C			**Benzene 50 ppb at 4 °C**		
Month	Benzene (ppb)	Σ Degradation %	Month	Benzene (ppb)	Σ Degradation %
0	50	0	0	50	0
1	3.9	92	1	33.2	33
2	6.1	88	2	4.4	91
3	5.4	89	3	3.2	93
4	6.0	88	4	4.1	92
5	4.3	91	5	0	100
6	0	100	6	0	100
Iron-Reducing Bacteria			**Iron-Reducing Bacteria**		
Benzene 200 ppb at 21 °C			**Benzene 200 ppb at 4 °C**		
Month	Benzene (ppb)	Σ Degradation %	Month	Benzene (ppb)	Σ Degradation %
0	200	0	0	200	0
1	3.5	98	1	26	87
2	18.4	91	2	6.3	97
3	4.3	98	3	9.0	95
4	12.6	94	4	4.1	98
5	6.5	97	5	8.3	96
6	3.9	98	6	5.4	98

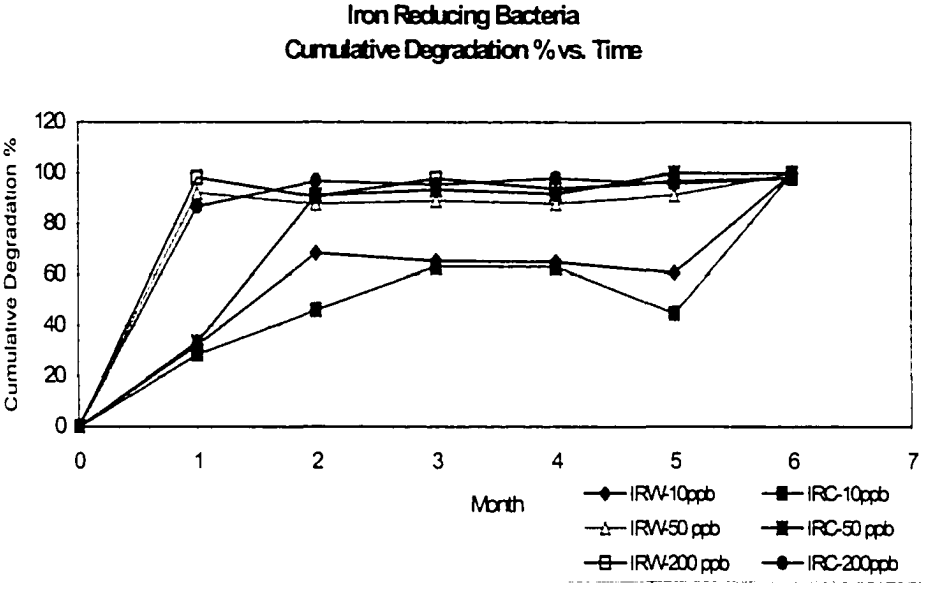

Figure 4.10: Cumulative degradation (%) over a period of six months under conditions favorable to iron-reducing bacteria. The percentages of benzene are the mean of the three replicates measured every month.
IRW-10 ppb = Iron-reducing conditions, amended with 10 ppb benzene at 21 °C
IRC-10 ppb = Iron-reducing conditions, amended with 10 ppb benzene at 4 °C
IRW-50 ppb = Iron-reducing conditions, amended with 50 ppb benzene at 21 °C
IRC-50 ppb = Iron-reducing conditions, amended with 50 ppb benzene at 4 °C
IRW-200 ppb = Iron-reducing conditions, amended with 200 ppb benzene at 21 °C
IRC-200 ppb = Iron-reducing conditions, amended with 200 ppb benzene at 4 °C

4.3.2 Identifying Intermediates

The GC/MS was used to identify intermediates in active serum bottles. Liquid samples were collected from bottles that produced detectable sulfide ions. Under sulfate-reducing conditions, phenol was detected during the analysis (data not shown). In addition, under iron-reducing conditions, both phenol and propionic acid were found in samples that contained detectable ferrous iron (data not shown).

4.3.3 Detection of Contamination

Ethylbenzene, p-xylene, and o-xylene were detected after the first month of incubation in both the experimental active and control bottles. The concentration of such compounds increased over the six month period and was around 2 times greater at 21 °C than at 4 °C. The highest concentrations were detected at four to six months in the controls. The concentration of p-xylene was higher than that of ethylbenzene or o-xylene. Figures 4.11-4.18 show the concentrations of benzene, ethylbenzene, p-xylene, and o-xylene in controls that contained mercuric chloride and autoclaved controls under sulfate-and iron-reducing conditions. Analyses of the controls done at 13 months showed that the concentrations of benzene, ethylbenzene, p-xylene, and o-xylene, were similar to those at 6 months, with a loss of only 4%. No further measurements were done.

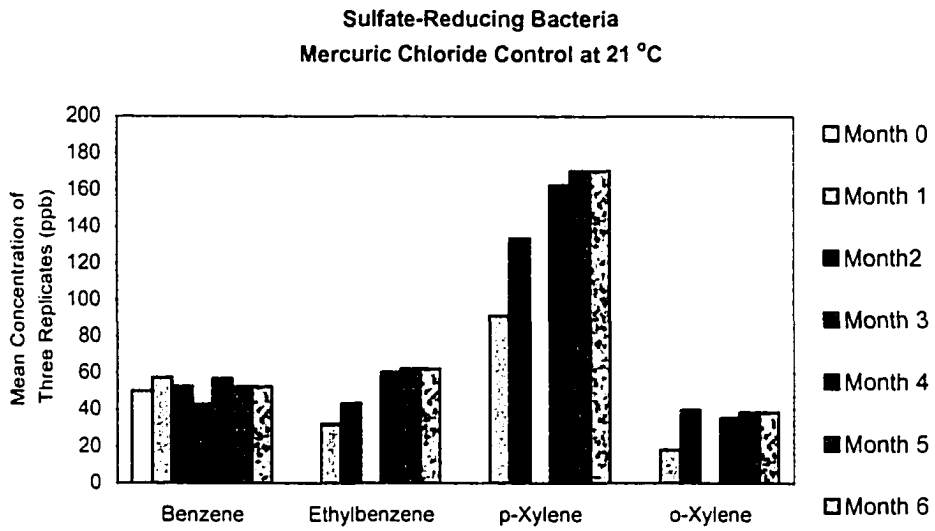

Figure 4.11: Mercuric chloride controls amended with 50 ppb of benzene under sulfate-reducing conditions over a period of six months at 21 °C. The missing data are due to no measurements being done. The initial benzene concentration was calculated.

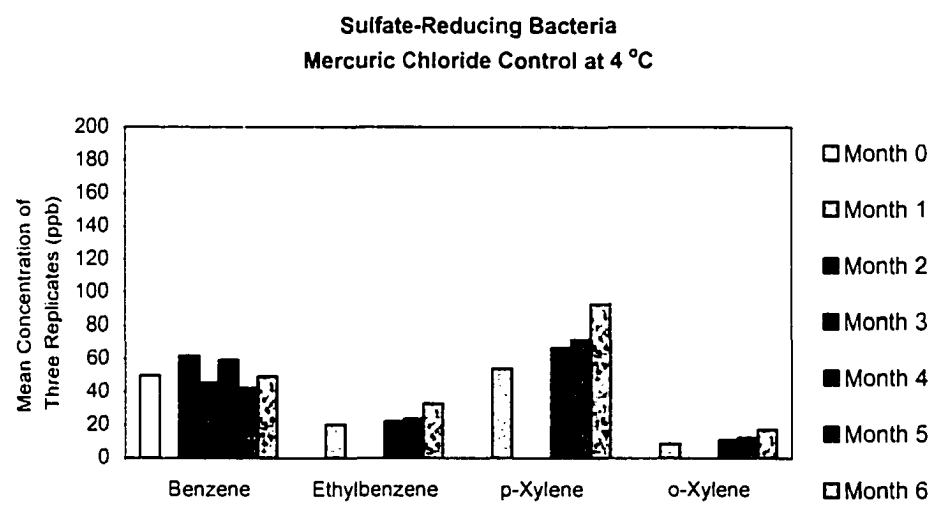

Figure 4.12: Mercuric chloride controls amended with 50 ppb of benzene under sulfate-reducing conditions over a period of six months at 4 °C. The missing data are due to no measurements being done. The initial benzene concentration was calculated.

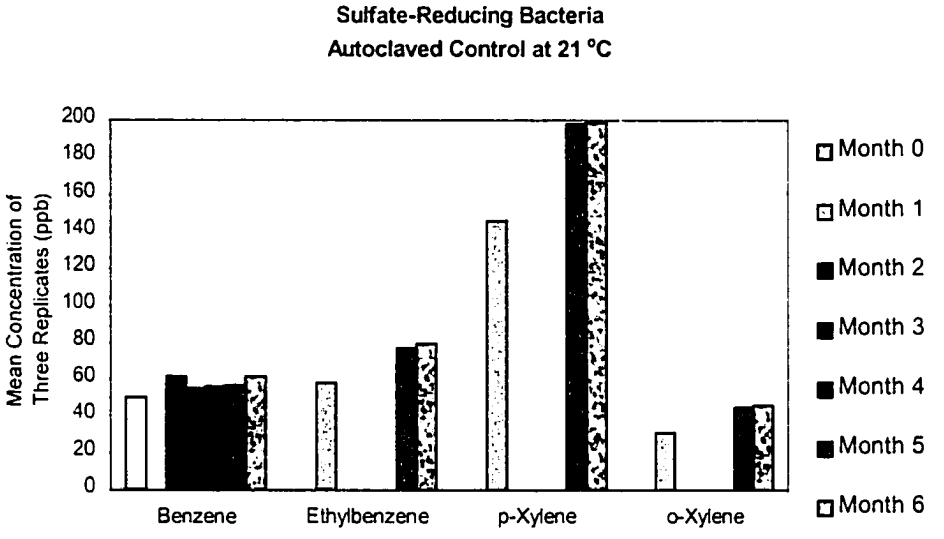

Figure 4.13: Autoclaved controls amended with 50 ppb of benzene under sulfate-reducing conditions over a period of six months at 21 °C. The missing data are due to no measurements being done. The initial benzene concentration was calculated.

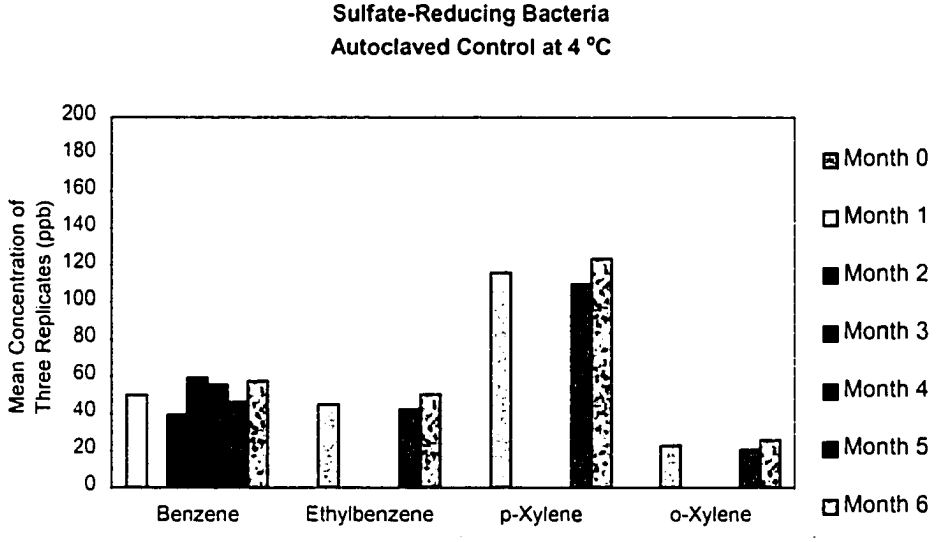

Figure 4.14: Autoclaved controls amended with 50 ppb of benzene under sulfate-reducing conditions over a period of six months at 4 °C. The missing data are due to no measurements being done. The initial benzene concentration was calculated.

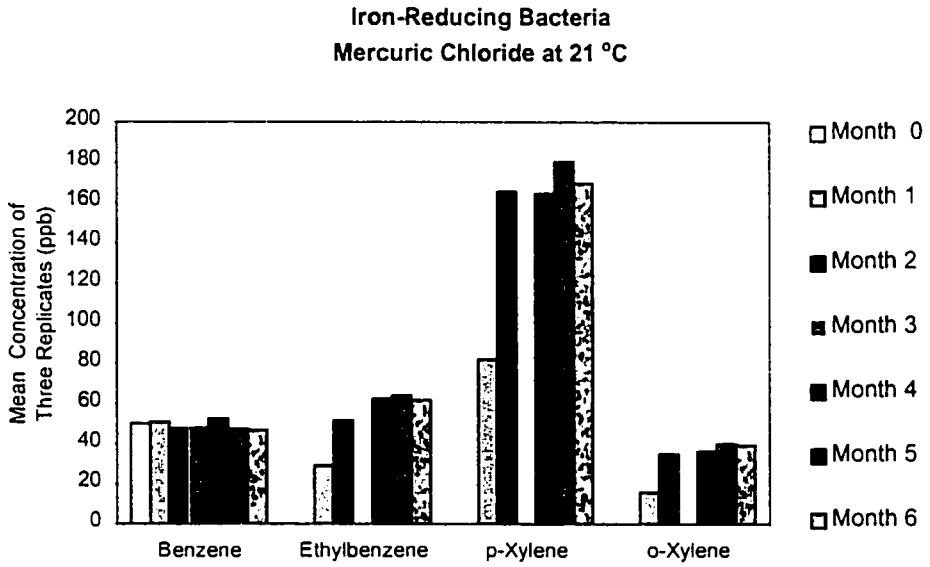

Figure 4.15: Mercuric chloride controls amended with 50 ppb of benzene under iron-reducing conditions over a period of six months at 21 °C. The missing data are due to no measurements being done. The initial benzene concentration was calculated.

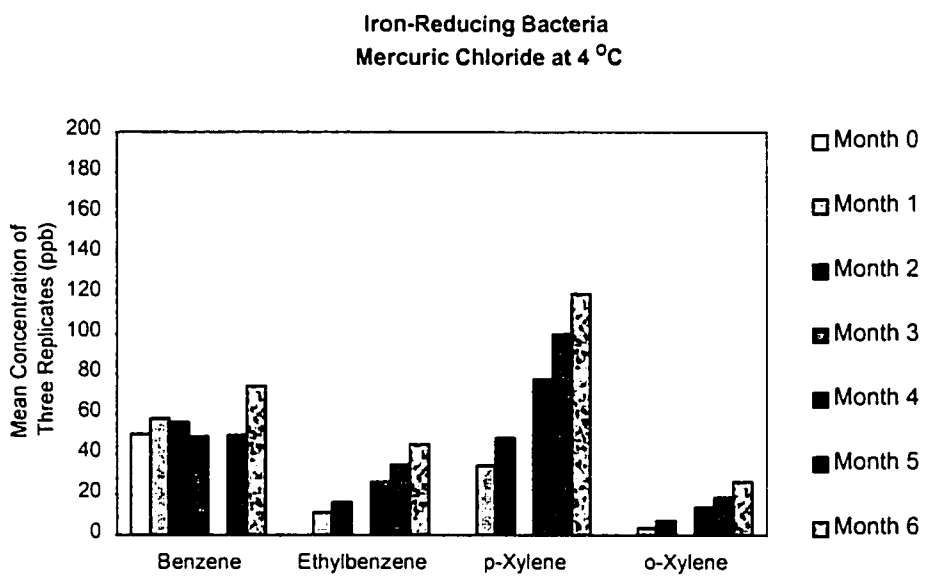

Figure 4.16: Mercuric chloride controls amended with 50 ppb of benzene under iron-reducing conditions over a period of six months at 4 °C. The missing data are due to no measurements being done. The initial benzene concentration was calculated.

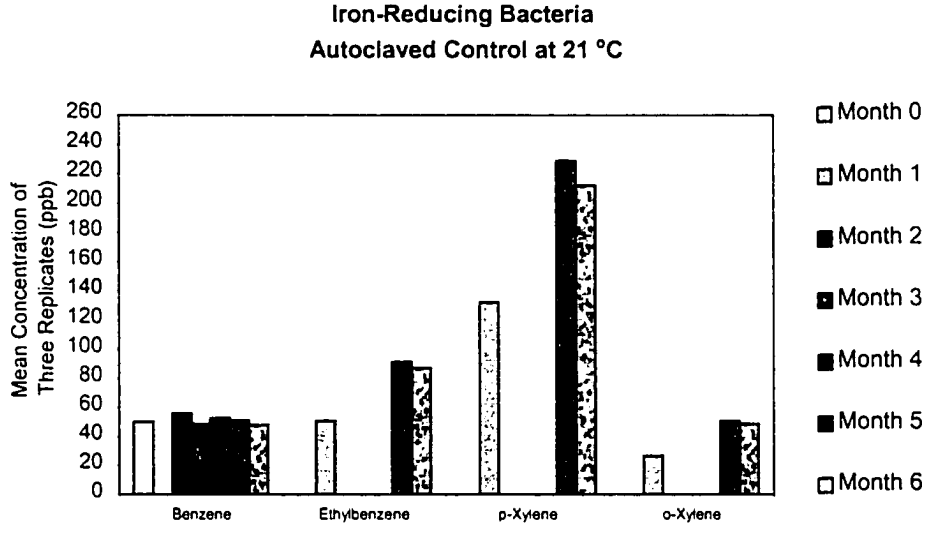

Figure 4.17: Autoclaved controls amended with 50 ppb of benzene under iron-reducing conditions over a period of six months at 21 °C. The missing data are due to no measurements being done. The initial benzene concentration was calculated.

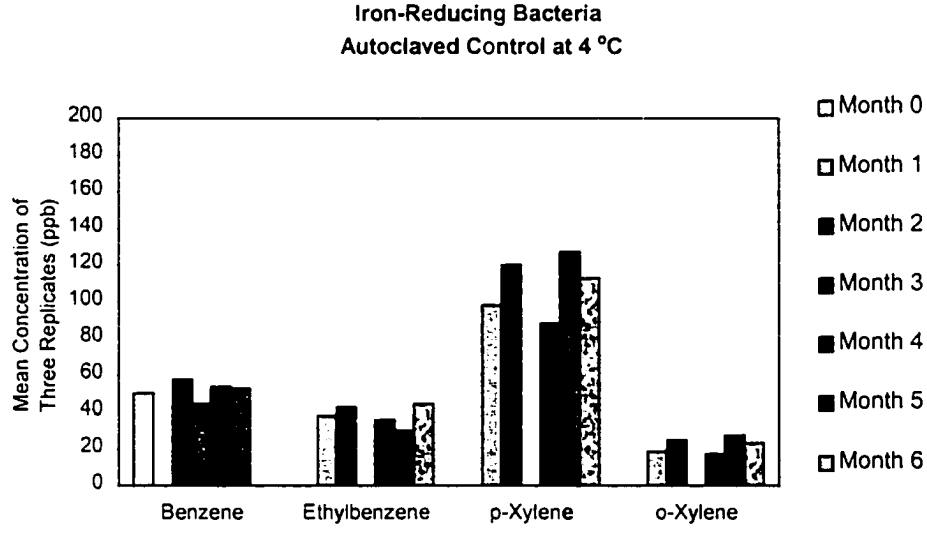

Figure 4.18: Autoclaved controls amended with 50 ppb of benzene under iron-reducing conditions over a period of six months at 4 °C. The missing data are due to no measurements being done. The initial benzene concentration was calculated.

The experimental bottles also showed an increase of ethylbenzene, p-xylene, and o-xylene over time. As in the controls, the p-xylene concentration was greater than that of ethylbenzene or o-xylene. The concentration fluctuated over a period of six months (Figures 4.19-4.30).

In order to assess the release of ethylbenzene, p-xylene, and o-xylene under sulfate-reducing conditions, 140 ml of fresh medium and 10 ml of an old medium were mixed in a 160-ml serum bottle amended with 250 ppb of benzene. The release of ethylbenzene, p-xylene, and o-xylene was measured over a period of 31 days at 4 °C. The benzene concentration initially decreased rapidly, within 18 days. Then the apparent rate of degradation slowed, and the concentration decreased to 4.5 ppb after 31 days (Figure 4.31).

Under iron-reducing conditions, ethylbenzene, p-xylene, and o-xylene were measured for an additional 190 days after the initial incubation period. The concentration of p-xylene decreased, to zero at 170 days. The concentrations of ethylbenzene and o-xylene started dropping when the concentration of p-xylene reached approximately 70 ppb under iron-reducing conditions. Both the ethylbenzene and o-xylene concentrations ultimately reached zero. No p-xylene, ethylbenzene and o-xylene were found over the remaining 20 days (Figure 4.32).

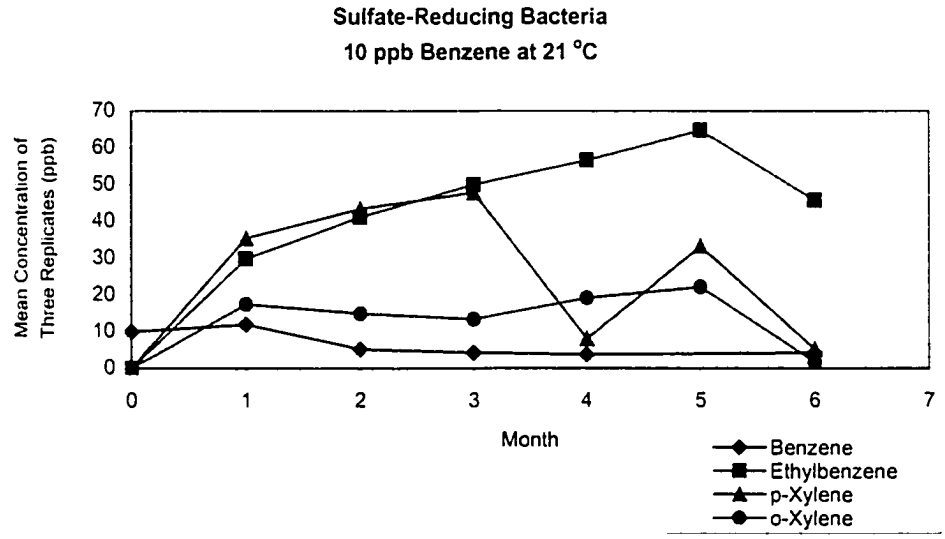

Figure 4.19: Biodegradation of 10 ppb of benzene and the concentrations of contaminants under sulfate-reducing conditions over a period of six months at 21 °C. The initial benzene concentration was calculated.

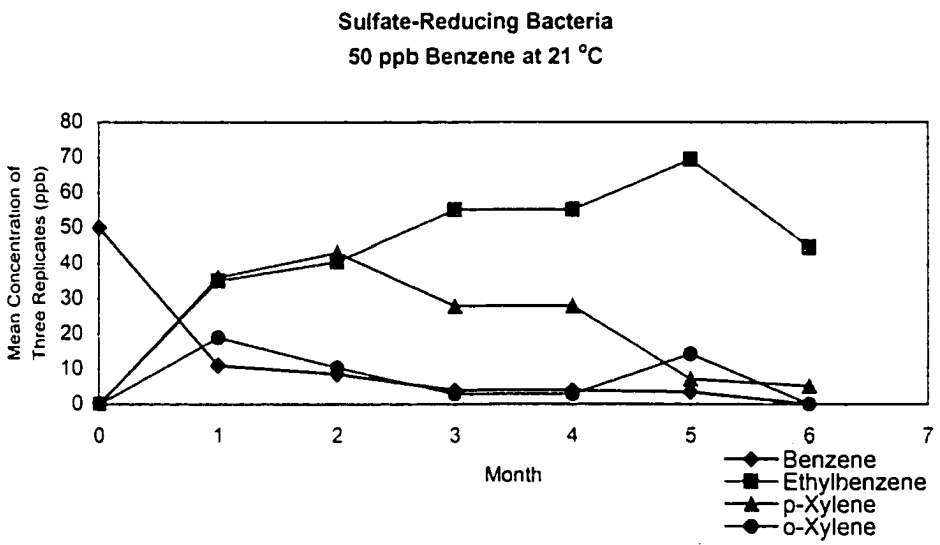

Figure 4.20: Biodegradation of 50 ppb of benzene and the concentrations of contaminants under sulfate-reducing conditions over a period of six months at 21 °C. The initial benzene concentration was calculated.

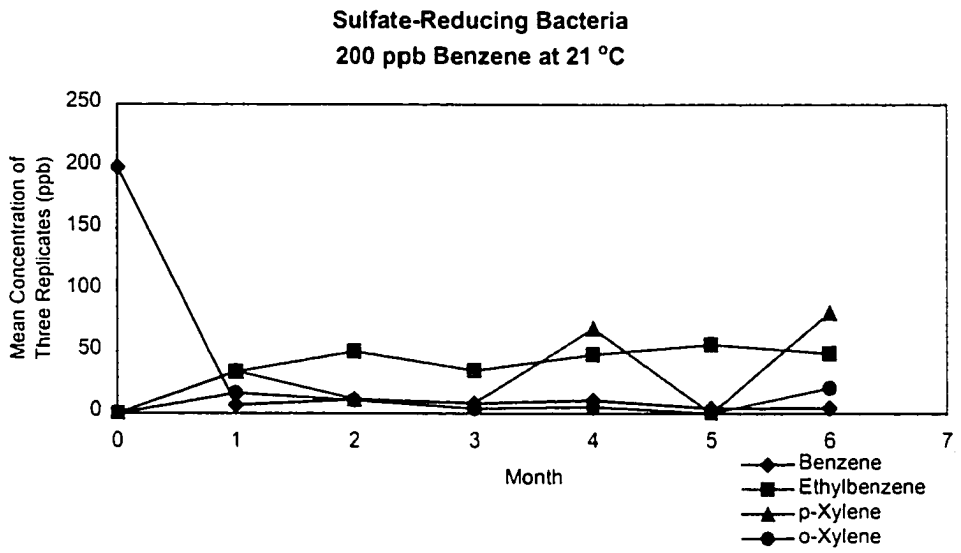

Figure 4.21: Biodegradation of 200 ppb of benzene and the concentrations of contaminants under sulfate-reducing conditions over a period of six months at 21 °C. The initial benzene concentration was calculated.

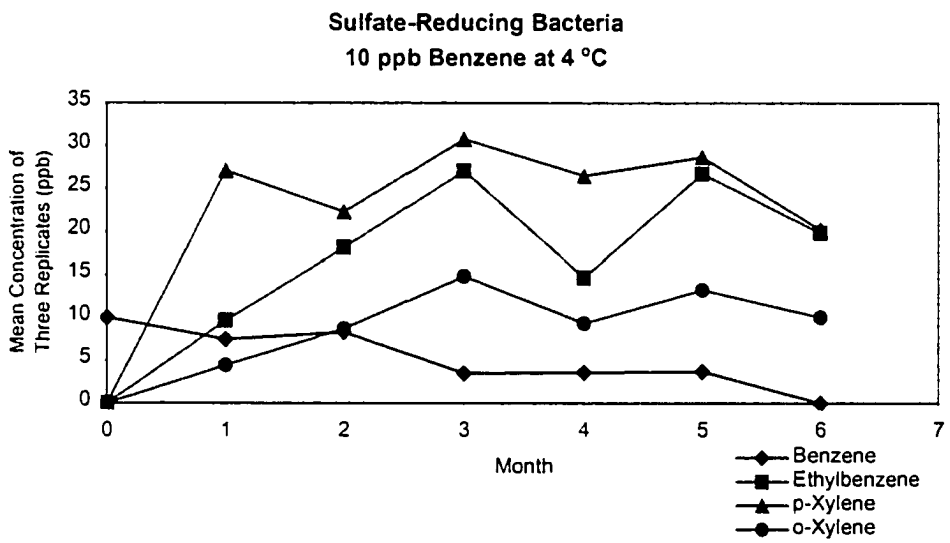

Figure 4.22: Biodegradation of 10 ppb of benzene and the concentrations of contaminants under sulfate-reducing conditions over a period of six months at 4 °C. The initial benzene concentration was calculated.

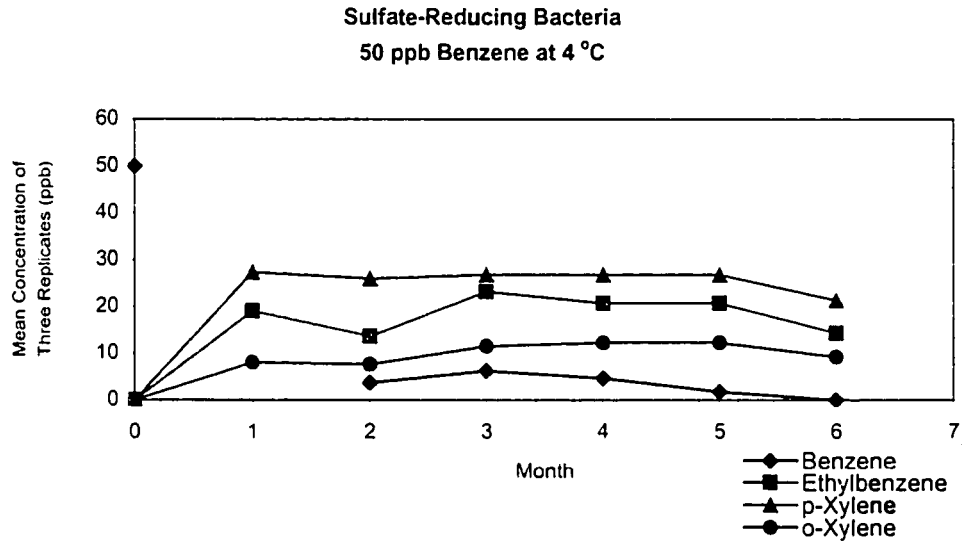

Figure 4.23: Biodegradation of 50 ppb of benzene and the concentrations of contaminants under sulfate-reducing conditions over a period of six months at 4 °C. The initial benzene concentration was calculated.

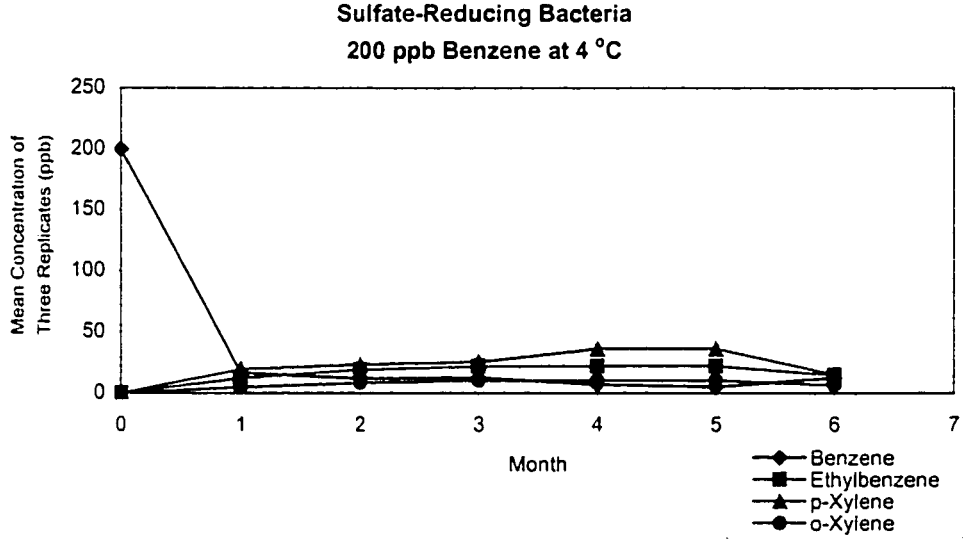

Figure 4.24: Biodegradation of 200 ppb of benzene and the concentrations of contaminants under sulfate-reducing conditions over a period of six months at 4 °C. The initial benzene concentration was calculated.

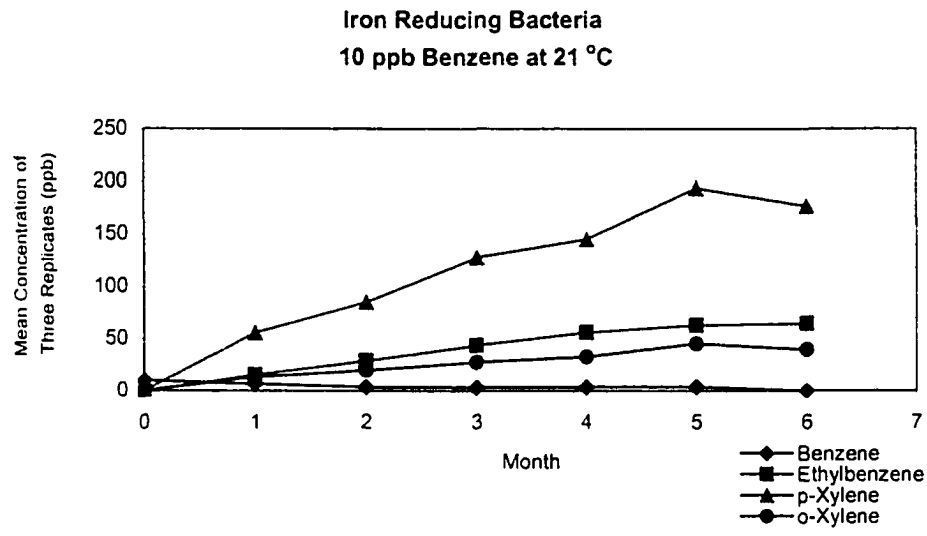

Figure 4.25: Biodegradation of 10 ppb of benzene and the concentrations of contaminants under iron-reducing conditions over a period of six months at 21 °C. The initial benzene concentration was calculated.

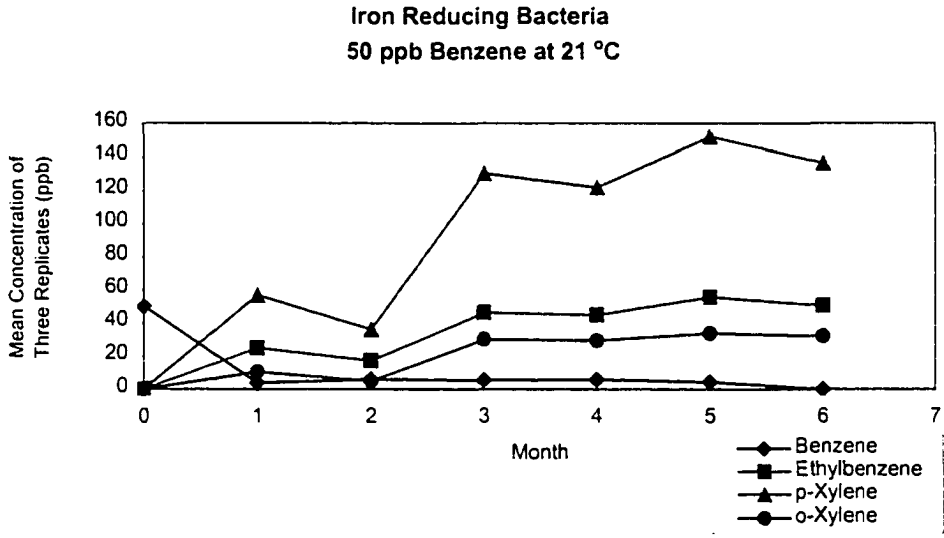

Figure 4.26: Biodegradation of 50 ppb of benzene and the concentrations of contaminants under iron-reducing conditions over a period of six months at 21 °C. The initial benzene concentration was calculated.

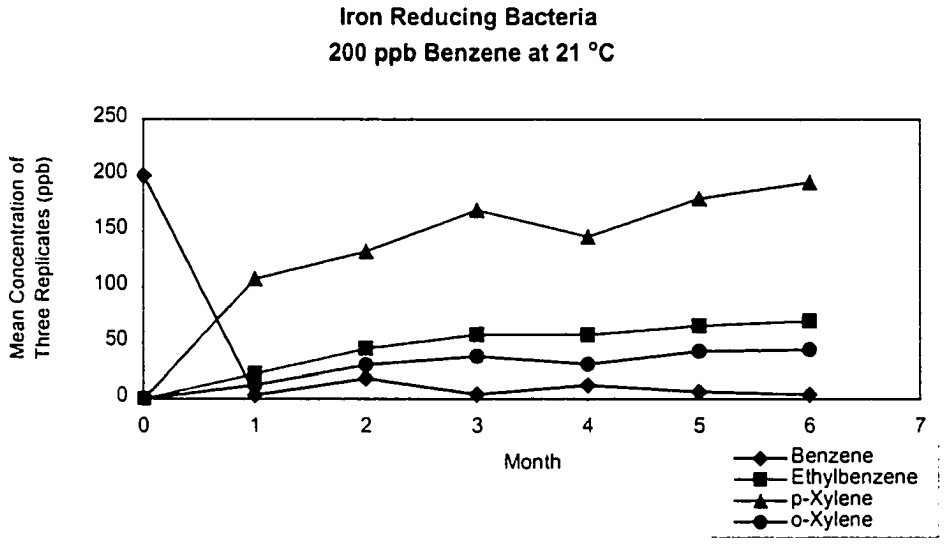

Figure 4.27: Biodegradation of 200 ppb of benzene and the concentrations of contaminants under iron-reducing conditions over a period of six months at 21 °C. The initial benzene concentration was calculated.

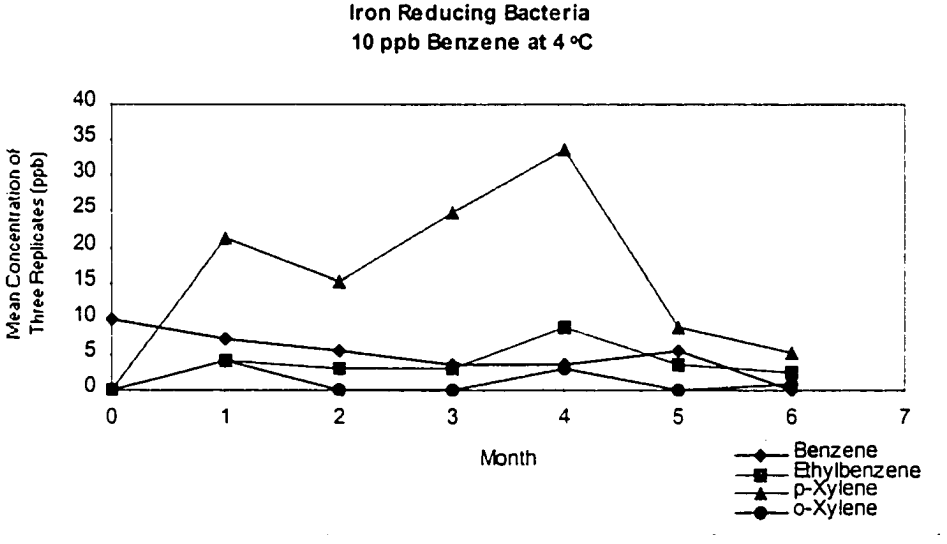

Figure 4.28: Biodegradation of 10 ppb of benzene and the concentrations of contaminants under iron-reducing conditions over a period of six months at 4 °C. The initial benzene concentration was calculated.

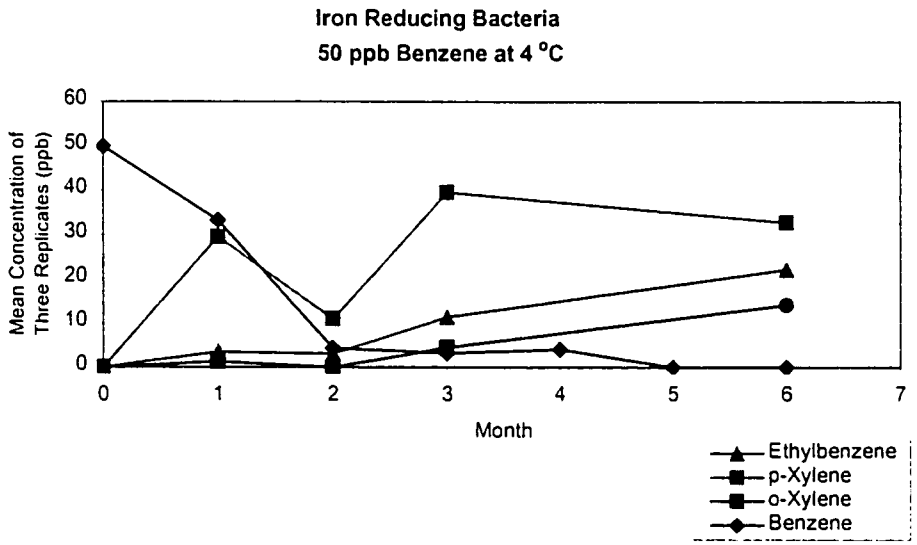

Figure 4.29: Biodegradation of 50 ppb of benzene and the concentrations of contaminants under iron-reducing conditions over a period of six months at 4 °C. The initial benzene concentration was calculated.

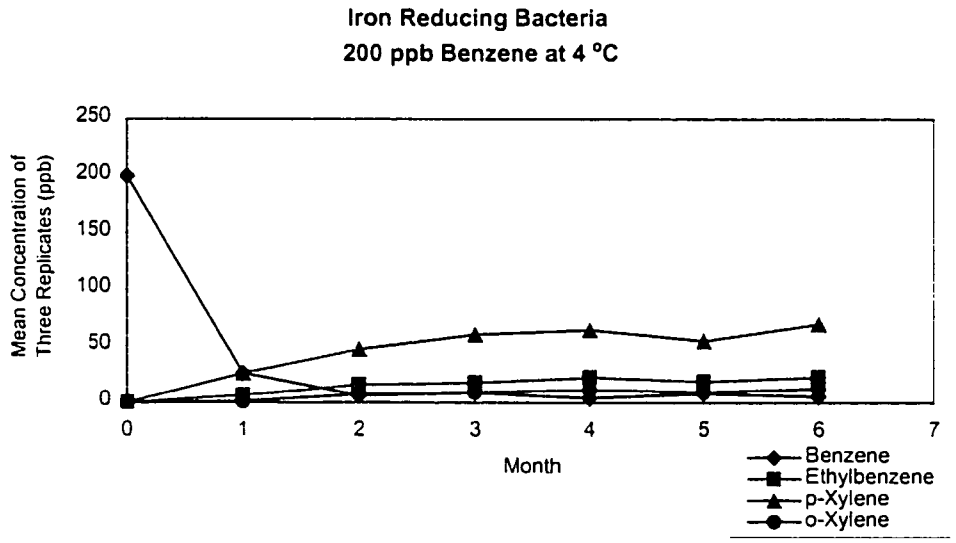

Figure 4.30: Biodegradation of 200 ppb of benzene and the concentrations of contaminants under iron-reducing conditions over a period of six months at 4 °C. The initial benzene concentration was calculated.

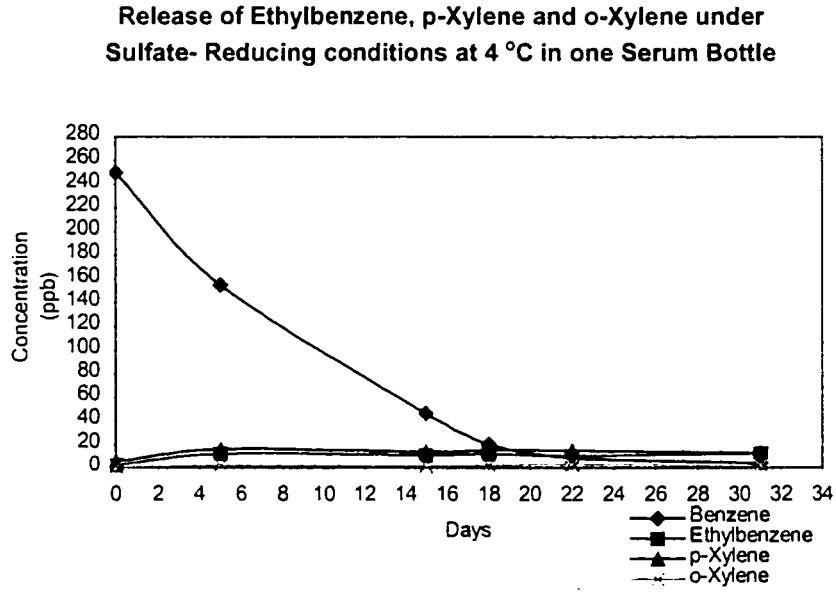

Figure 4.31: Release of ethybenzene, p-xylene, and o-xylene while benzene was degrading under sulfate-reducing conditions at 4 °C.

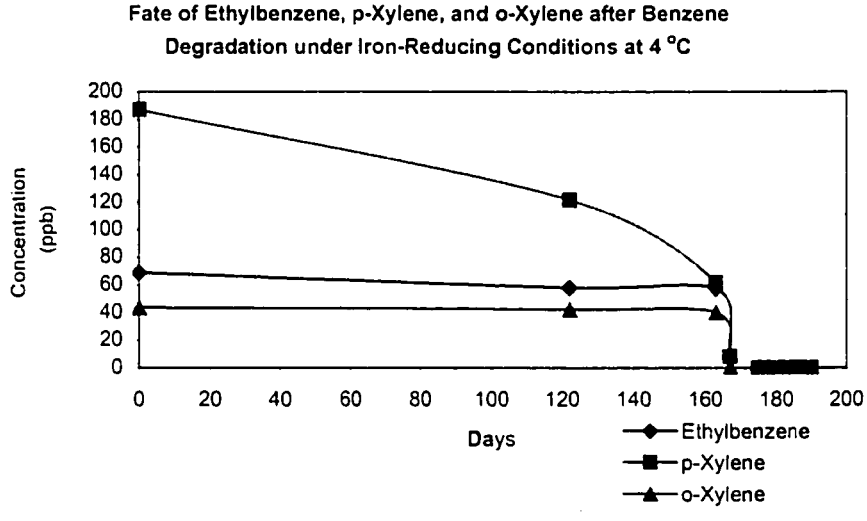

Figure 4.32: Fate of ethybenzene, p-xylene, and o-xylene after initial benzene degraded under iron-reducing conditions at 4 °C.

The rubber stoppers used on the serum bottles appeared to be the source of the ethylbenzene, p-xylene and o-xylene. To further investigate

the rubber stoppers as the source of the contaminants, four additional serum bottles were filled with autoclaved distilled water. Two of the four bottles were amended with 35 ppb benzene. Two were kept at 4 °C and the other two were kept at 21 °C. All were subjected to BTEX analysis over a period of 190 days. The release of ethylbenzene, p-xylene, and o-xylene from the butyl rubber stoppers was more than 50% greater at 21 °C than at 4 °C. The release of these compounds at 21 °C was about the same in the autoclaved distilled water serum bottle with distilled water, alone, and the bottle with distilled water and benzene (Figure. 4.33). Since the concentrations were similar to those in experimental bottles, and no plausible source existed except the stoppers in bottles containing distilled water, the stopper was confirmed as the source.

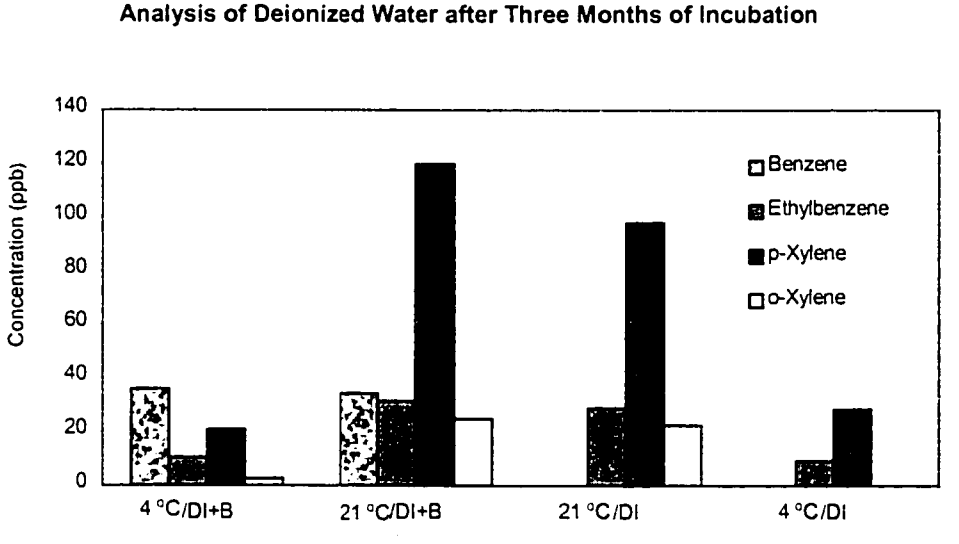

Figure 4.33: Concentration of ethylbenzene, p-xylene, and o-xylene after three months of incubating autoclaved deionized water (DI), amended and unamended with benzene (B), at 21 °C and at 4 °C.

4.3.4 Effect of Alternate Electron Acceptors

4.3.4.1 Sulfate Reduction Results

Dissolved sulfide ions were detected in some of the experimental bottles, but not in the controls, after the fourth month. At four months, 0.15 mg/l of sulfide was detected at 21 °C in one serum bottle out of 3 that had been amended with 10 ppb of benzene. Sulfide was also detected at 21 °C during the fourth month (0.95 and 0.5 mg/l) in 2 serum bottles, and the sixth month (0.57, 0.71, 0.7 mg/l) in the 3 serum bottles that had been amended with 200 ppb of benzene (Figures 4.34-4.39). Sulfide was not detected during the fifth month. Sulfide was not detected in any of the bottles, whether amended with 10, 50, or 200 ppb of benzene, at 4 °C. A black precipitate was observed in the bottles that had been amended with 200 ppb benzene, beginning in the fourth month at 21 °C and during the sixth month at 4 °C. Sulfate concentration remained above 1600 (mg/l) except during the fourth month for 10 and 200 ppb initial concentrations incubated at 21 °C (Figures 4.34 and 4.36).

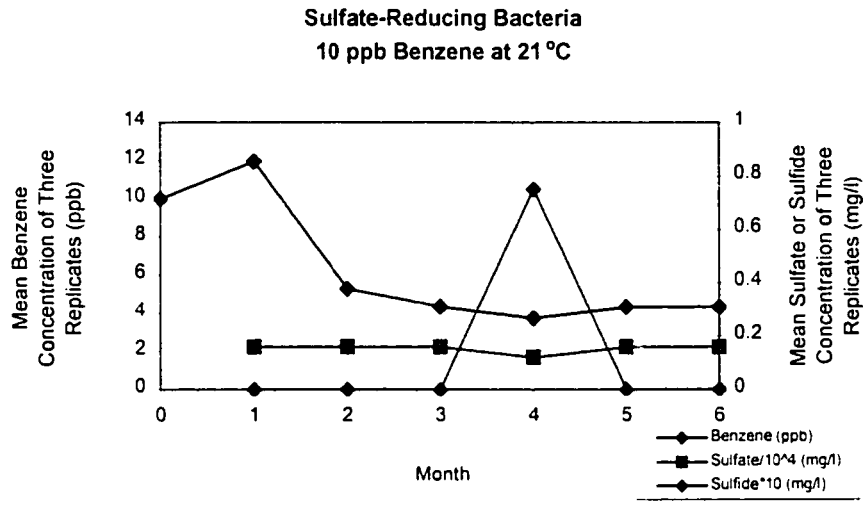

Figure 4.34: Concentration of sulfide ions during the biodegradation of 10 ppb of benzene under sulfate-reducing conditions over a period of six months at 21 °C. Sulfate values were divided by 10^4, and sulfide values were multiplied by 10. The initial benzene concentration was calculated.

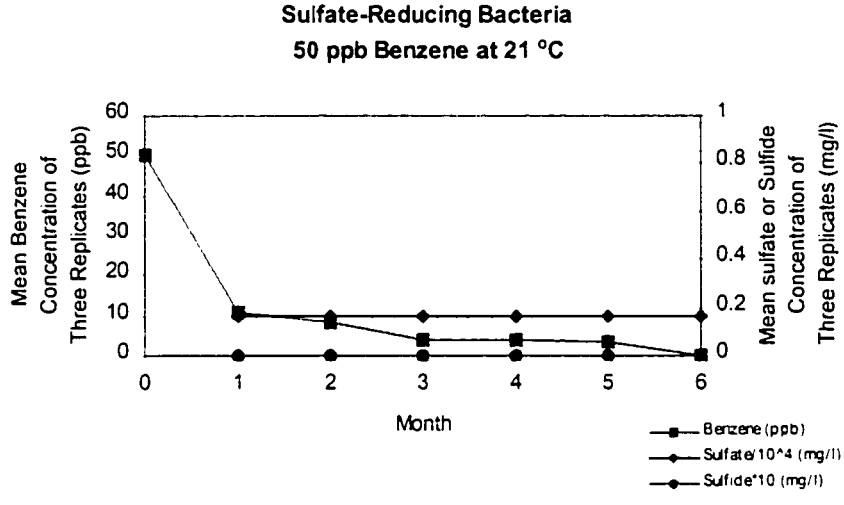

Figure 4.35: Concentration of sulfide ions during the biodegradation of 50 ppb of benzene under sulfate-reducing conditions over a period of six months at 21 °C. Sulfate values were divided by 10^4, and sulfide values were multiplied by 10. The initial benzene concentration was calculated.

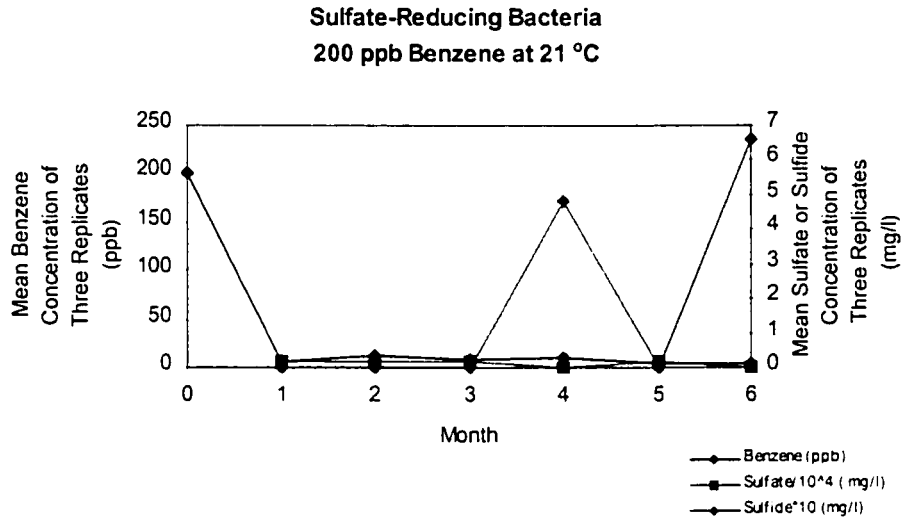

Figure 4.36: Concentration of sulfide ions during the biodegradation of 200 ppb of benzene under sulfate-reducing conditions over a period of six months at 21 °C. Sulfate values were divided by 10^4, and sulfide values were multiplied by 10. The initial benzene concentration was calculated.

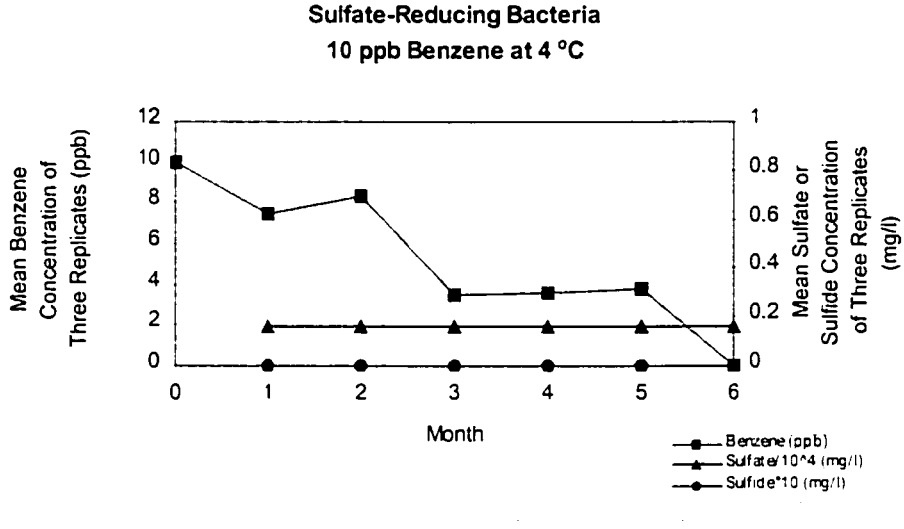

Figure 4.37: Concentration of sulfide ions during the biodegradation of 10 ppb of benzene under sulfate-reducing conditions over a period of six months at 4 °C. Sulfate values were divided by 10^4, and sulfide values were multiplied by 10. The initial benzene concentration was calculated.

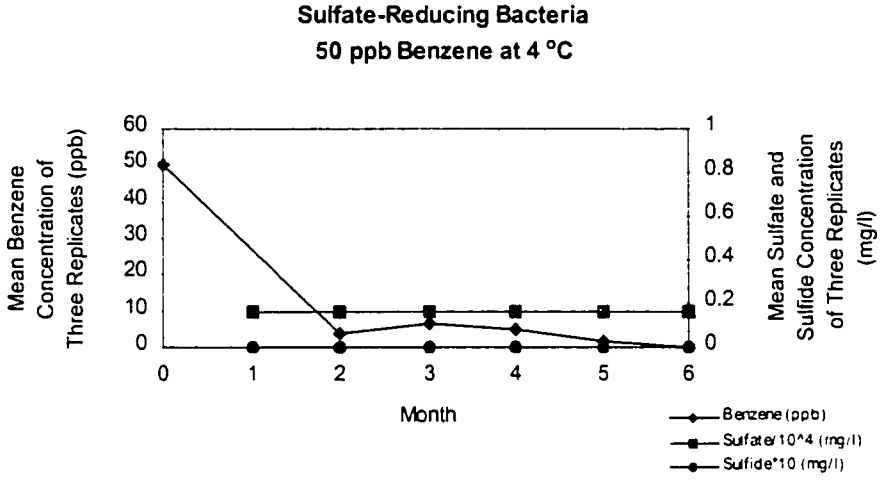

Figure 4.38: Concentration of sulfide ions during the biodegradation of 50 ppb of benzene under sulfate-reducing conditions over a period of six months at 4 °C. Sulfate values were divided by 10^4, and sulfide values were multiplied by 10. The initial benzene concentration was calculated.

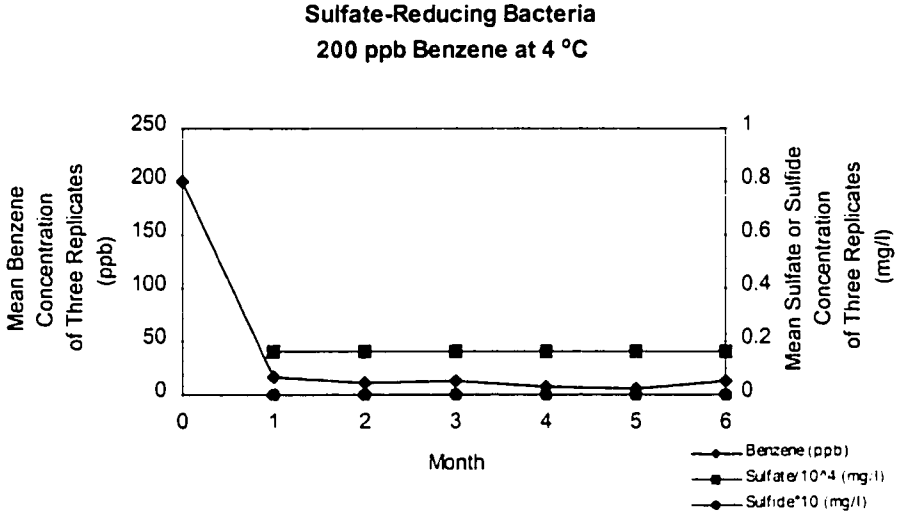

Figure 4.39: Concentration of sulfide ions during the biodegradation of 200 ppb of benzene under sulfate-reducing conditions over a period of six months at 4 °C. Sulfate values were divided by 10^4, and sulfide values were multiplied by 10. The initial benzene concentration was calculated.

4.3.4.2 Iron-Reduction Results

Dissolved ferrous iron was detected in the experimental active bottles but not in the controls. Ferrous iron was detected at one month at 21 °C, when initial benzene concentrations were 10, 50, and 200 ppb (Figures 4.40-4.42). The concentration of ferrous ions in the solution was variable at 4 °C (Fig 4.43-4.45). Dissolved ferrous iron was detected in the fourth month when the initial benzene concentration was 10 ppb, the third month when the initial benzene concentration was 50 ppb, and the second month when the initial benzene concentration was 200 ppb (Figures 4.43-4.45).

The concentration of dissolved ferrous ions was high after the fourth month at both 21 °C and 4 °C, coinciding with the accumulation of ethylbenzene, p-xylene, and o-xylene released from the butyl rubber stoppers. The dissolved ferrous iron concentrations at one month were zero in the serum bottles that were amended with 10, 50, and 200 ppb of benzene at 4 °C. It remained zero during the second month in the bottles that were amended with 10 and 50 ppb of benzene but averaged 1950 ppb in the bottles that were amended with 200 ppb of benzene at 4 °C.

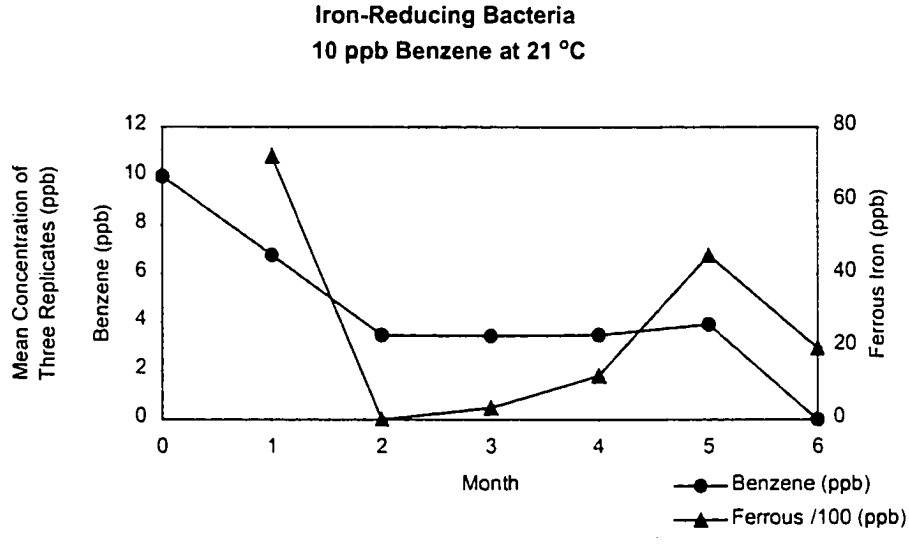

Figure 4.40: Concentration of ferrous ions in solution as 10 ppb of benzene was degraded at 21 °C. The initial benzene concentration was calculated. The ferrous iron concentration was divided by 100.

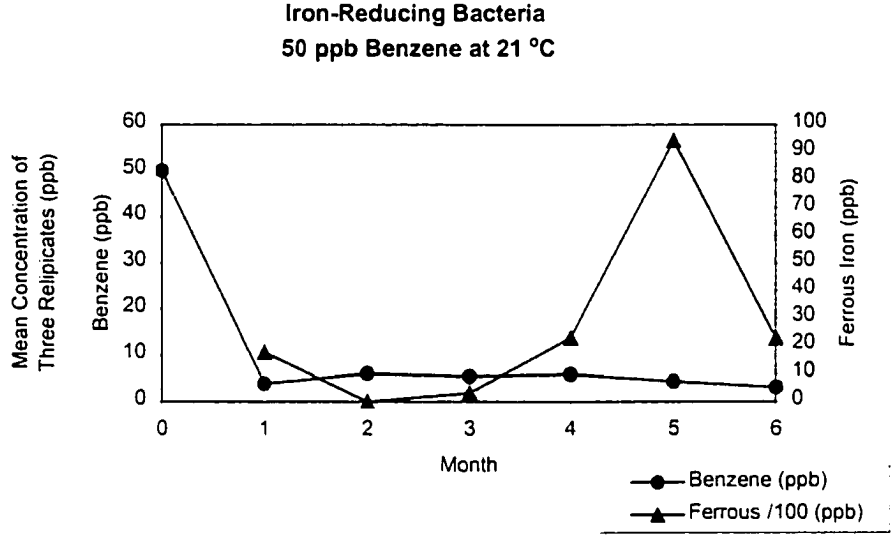

Figure 4.41: Concentration of ferrous ions in solution as 50 ppb of benzene was degraded at 21 °C. The initial benzene concentration was calculated. The ferrous iron concentration was divided by 100.

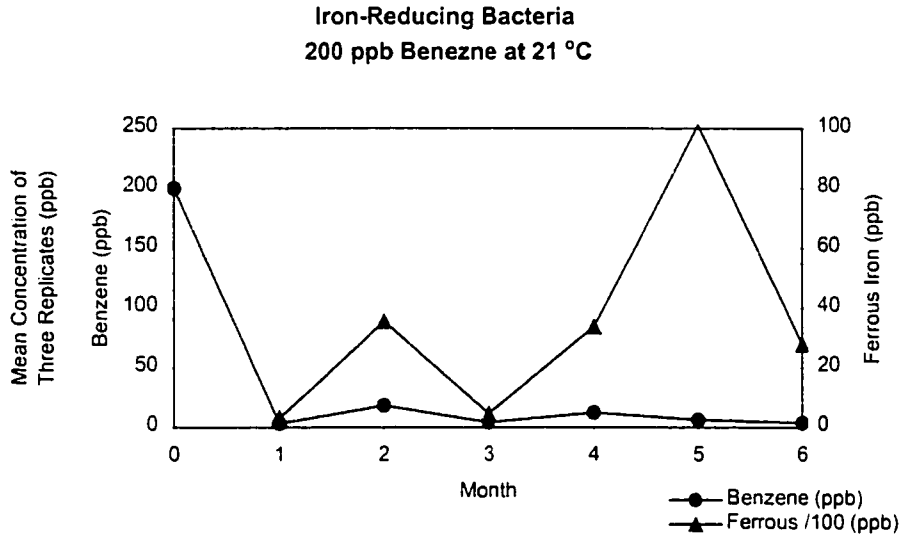

Figure 4.42: Concentration of ferrous ions in solution as 200 ppb benzene was degraded at 21 °C. The initial benzene concentration was calculated. The ferrous iron concentration was divided by 100.

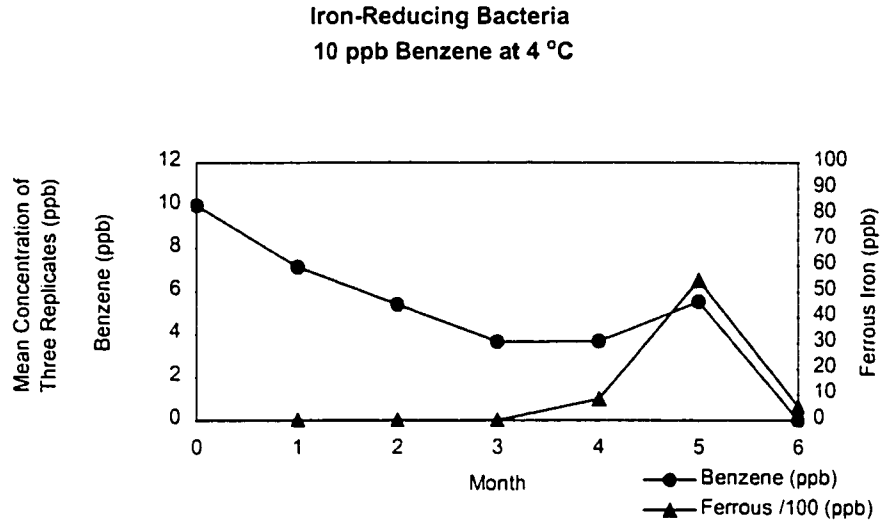

Figure 4.43: Concentration of ferrous ions in solution as 10 ppb benzene was degraded at 4 °C. The initial benzene concentration was calculated. The ferrous iron concentration was divided by 100.

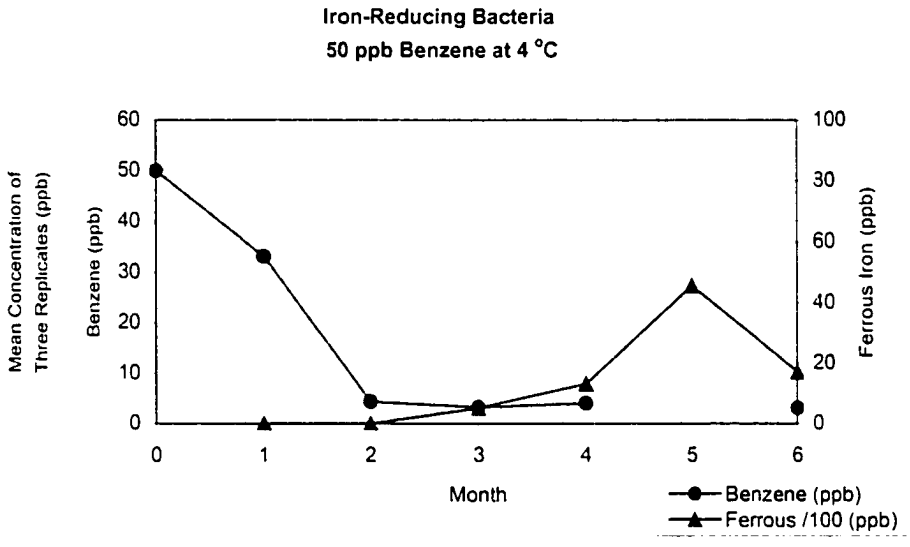

Figure 4.44: Concentration of ferrous ions in solution as 50 ppb benzene was degraded at 4 °C. The initial benzene concentration was calculated. The ferrous iron concentration was measured divided by 100.

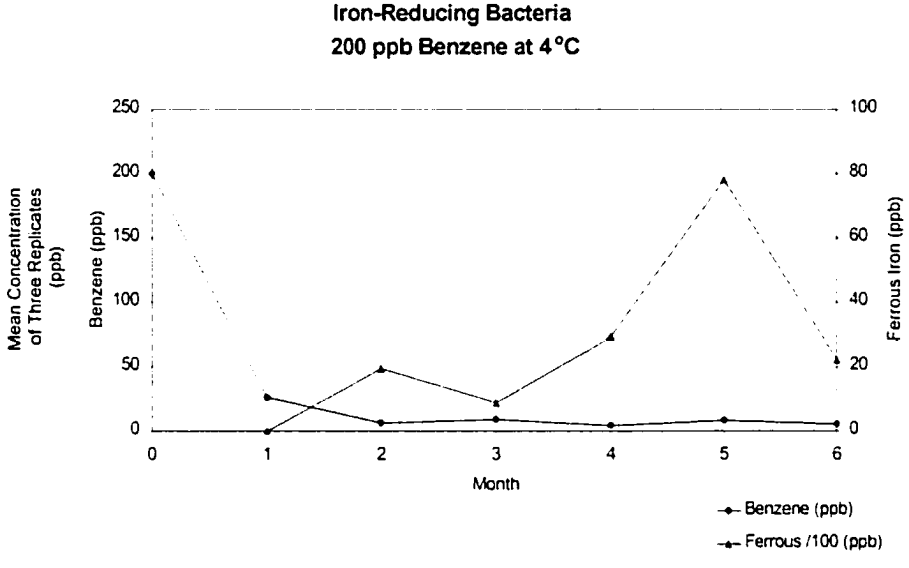

Figure 4.45: Concentration of ferrous ions in solution as 200 ppb benzene was degraded at 4 °C. The initial benzene concentration was calculated. The ferrous iron concentration was divided by 100.

4.3.4.3 Benzene Biodegradation over 30 Days

In most of the experiments, the extent of benzene biodegradation was not measured until after one month. In Figure 4.46 biodegradation of 25 ppb of benzene over a period of 35 days is shown, for iron-reducing conditions at 4 °C. Benzene was completely degraded over a period of 35 days, with a lag of 15 days.

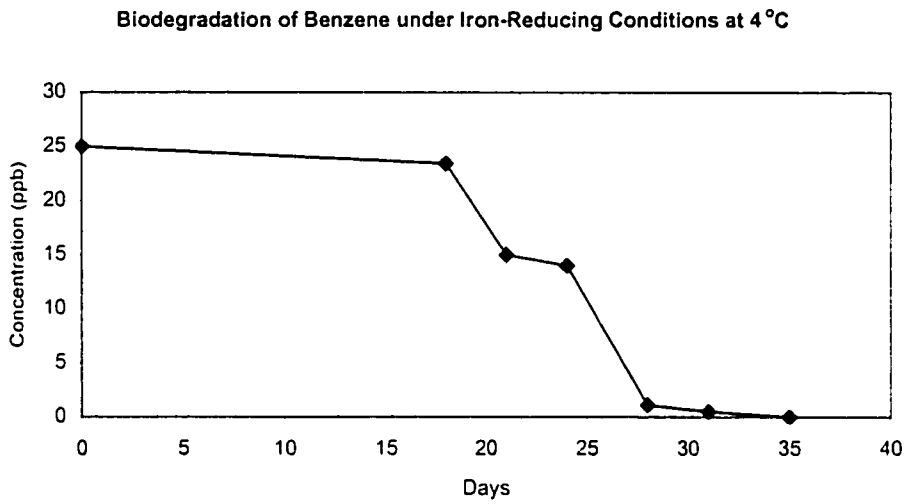

Figure 4.46: Biodegradation of 25 ppb of benzene at 4 °C in a period of 35 days.

4.3.5 Anaerobic Growth

An increase in the amount of protein is an indication of an increase in cell mass. However, in the experimental bottles, the protein fluctuated over time and there was no evidence of net growth after the first month. In general, under sulfate-reducing conditions, protein concentration decreased after the second month, when benzene concentrations had decreased to low levels. The protein concentration increased again

during the fourth month. The absorbance of the controls had a value approximately 1 on the protein standard curve. This value was subtracted from the protein concentration values of the experimental bottles. Also, as mentioned previously, in controls there were no bacterial cells detected under the microscope. The protein concentration data are summarized in Table 4.3.

Table 4.3: The range of protein concentration under sulfate-reducing conditions over the period of six months at 21 °C and at 4 °C.

Month	Protein (μg/ml)	Protein (μg/ml)
	21 °C	4 °C
1	1.3-3.4	1.3-3.4
2	2.3-3.3	2.3-3.3
3	1.6-2.8	1.6-2.8
4	7.4-13	0.9-7.3
5	1.4-6.3	1.4-6.3
6	2-7.4	2-7.4

The protein concentration data under iron-reducing conditions are summarized in Table 4.4. As under sulfate-reducing conditions, there was no clear temporal trend.

Table 4.4: The range of the protein concentration under iron-reducing conditions over the period of six months at 21°C and at 4 °C.

Month	Protein (μg/ml)	Protein (μg/ml)
	21 °C	4 °C
1	1.3-1.74	1.3-1.74
2	3.7-8.7	0.4-1.3
3	0.1-.97	0.97-2.07
4	1.1-2.47	4.35-5.17
5	0.17-1.74	0.57-1.94
6	0.1.1-1.6	0.8-1.57

The bacterial consortium growing on benzene was heterogeneous. Rod-shaped cells (bacilli) were dominant over spherical cells (cocci) (Figures 4.47-4.50). The observed cells were Gram-negative. In addition, microscopic observation confirmed the presence of capsules surrounding some of the cells. After exposing the cells to severe temperatures, -20 °C and 80 °C, for a period of one hour at each temperature, microscopic observation confirmed the presence of spores.

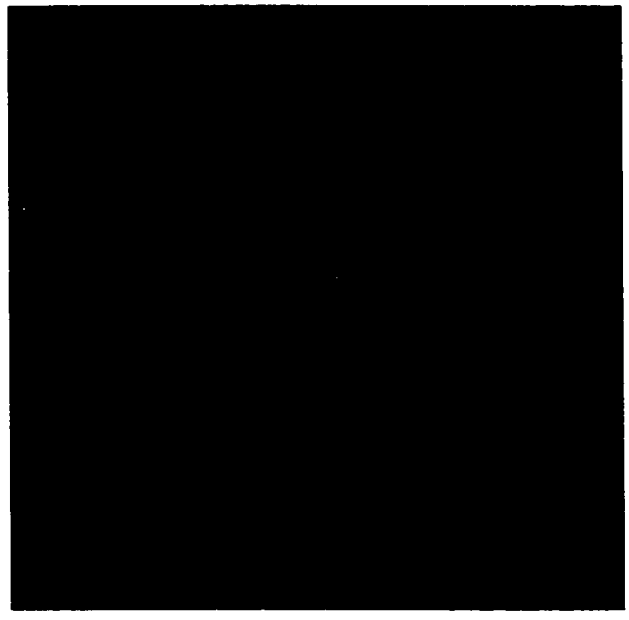

Figure 4.47: Rod-shaped cells (bacilli) were dominant over spherical cells (cocci) under sulfate-reducing conditions at 21 °C.

Figure 4.48: Rod-shaped cells (bacilli) were dominant over spherical cells (cocci) under sulfate-reducing conditions at 4 °C.

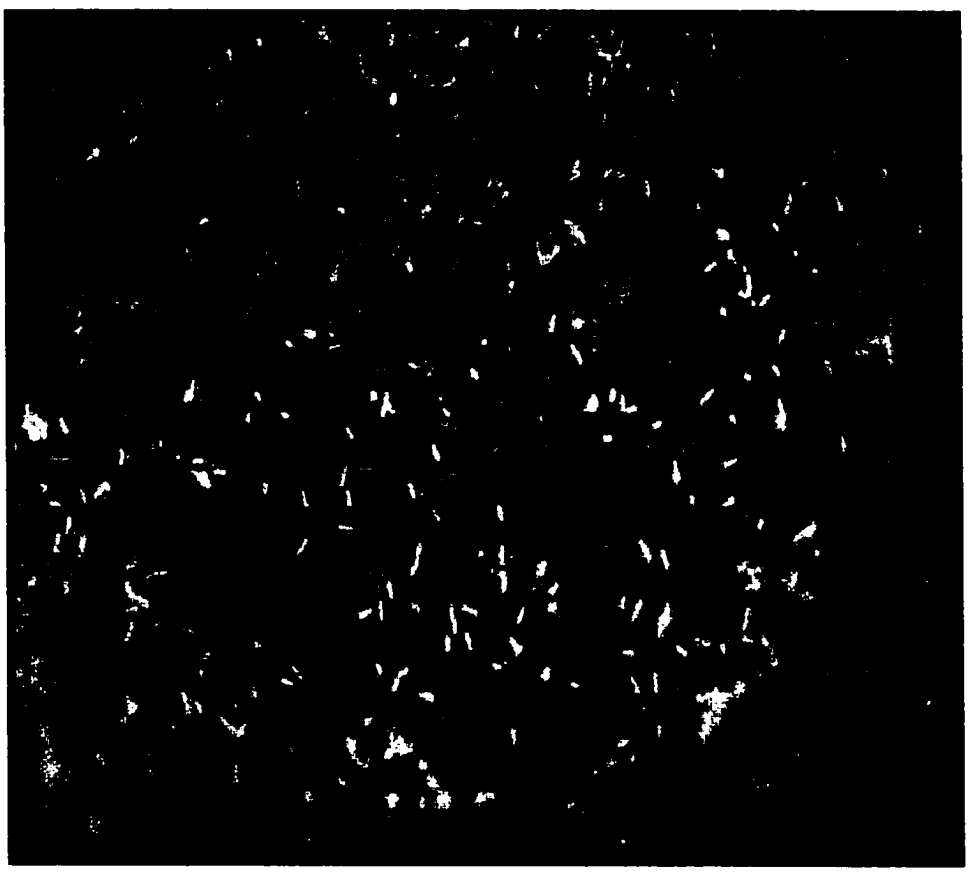

Figure 4.49: Rod-shaped cells (bacilli) were dominant over spherical cells (cocci) under iron-reducing conditions at 21 °C.

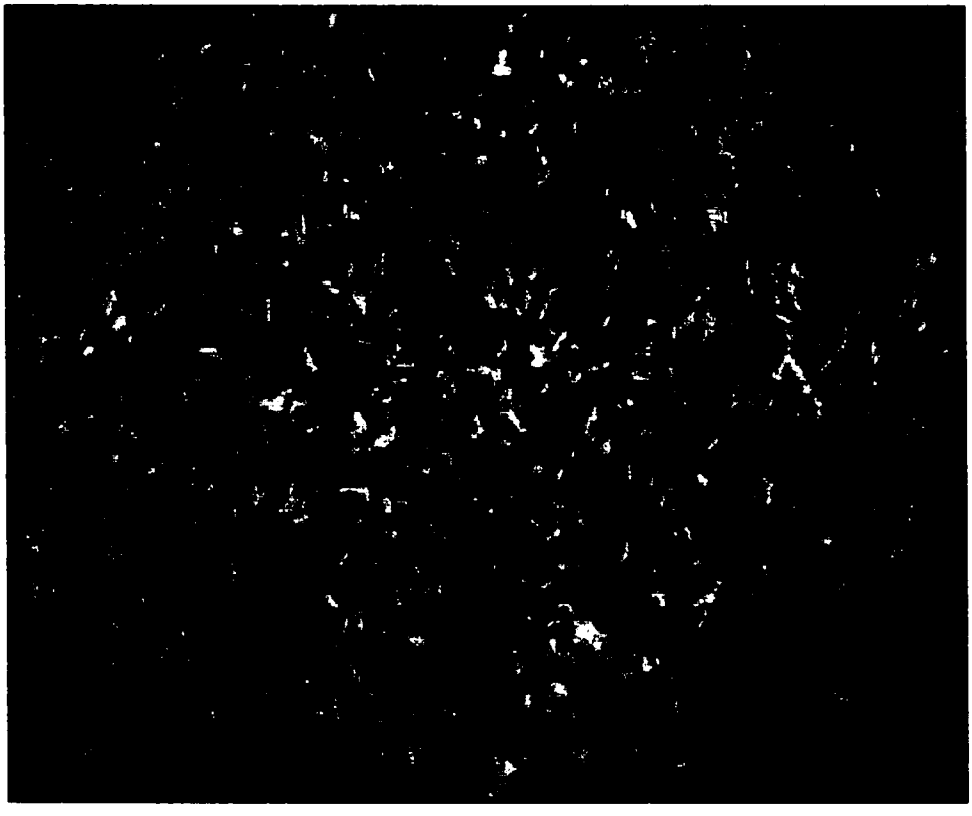

Figure 4.50: Rod-shaped cells (bacilli) were dominant over spherical cells (cocci) under iron-reducing conditions at 4 °C.

Also, under iron-reducing conditions, the microorganisms were able to survive starvation, without addition of benzene or other organic compounds, for at least seven months. The rate of benzene degradation at 4 °C after starvation was slow for the initial 19 days, but subsequently was faster. Benzene degraded completely after 30 days (Figure 4.51).

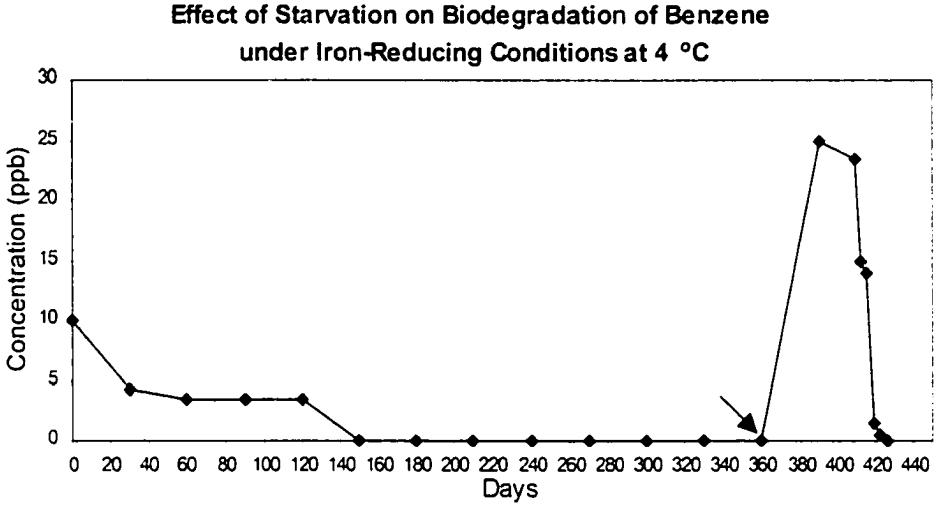

Figure 4.51: Biodegradation of benzene after starvation at 4 °C. The initial benzene concentration was calculated. Arrow indicates benzene addition on day 360.

4.4 Discussion

Anaerobic biodegradation of benzene at temperatures above 10 °C is known to occur (Alvarez and Vogel, 1991; Alvarez et al., 1991; Hunt et al. 1994; Edwards and Gribic`-Galic`, 1992; Lovely et al. 1995; Borden at al. 1997; Kazumi et al. 1997; Anderson et al. 1998), but has not been previously observed at temperatures below 10 °C. The benzene

data presented in this chapter are based on the mean of three replicates as done by Hutchins et al., 1991; Lovely and Phillips, 1994; Lovely et al., 1994; Anderson et al., 1998. In this work, it was shown that benzene could be degraded at 21 °C and at 4 °C under strictly anaerobic conditions in the presence of both sulfate or ferric iron. Benzene was also degraded under similar conditions but with neither sulfate nor ferric iron in the medium (See Chapter Five). This shows that fermentation is probably the main process in removing benzene. The only difference in data from microcosms with and without sulfate or ferric iron was that benzene degradation was usually less complete in those without. One plausible explanation for this observation is that, without the ferric iron or sulfate as terminal electron acceptor, some fermentation intermediates accumulated in concentrations that inhibited benzene metabolism. However, the refractory concentration was consistently small, less than 10 ppb.

Substantial benzene removal by non-biological processes can be ruled out. At the end of the six month period, there was only about 5% loss of benzene in the autoclaved or poisoned control bottles. Loss of benzene due to adsorption to the precipitate can't be ruled out based on the controls, since the precipitate did not form in the controls. However, the precipitate did not form in all of the experimental bottles, and benzene disappeared from the biologically active bottles regardless of the presence or absence of precipitate. Also, Gribic `-Galic` and Vogel (1987) noticed

that there was no difference in the dissolved radiolabeled ^{14}C activity between undisturbed and shaken culture fluids during anaerobic degradation of benzene at room temperature. They therefore suggested that very little substrate was adsorbed to FeS that precipitated as the result of production of ferrous ions.

The transformation pathway of benzene apparently includes ring oxidation. In this research, GC/MS was used to detect, qualitatively, transient intermediates. A trace amount of phenol was detected in the biologically active benzene microcosms that was not detected in the controls. Phenol was detected under both iron-and sulfate-reducing conditions. Similar findings were reported by Gribic`-Galic` and Vogel (1987), where phenol was detected as an intermediate during the transformation of benzene under methanogenic conditions. However, Lovely at al. (1995) did not detect any intermediates when benzene was degraded under sulfate reducing conditions. The anaerobic biodegradation of phenol into gaseous products has been well established in the literature. Evans (1977), Gribic`-Galic` and Vogel (1987) and Lovely and Lonergan (1990) have all shown reaction pathways for the transformation of phenol under anaerobic conditions (Figures 2.3-2.5).

The observed stoichiometry of the overall reaction of benzene was not compared to the theoretically predicted stoichiometry, because benzene was clearly not the only carbon source. The release of

ethylbenzene, p-xylene, and o-xylene from the butyl rubber stoppers (Figure 4.33) made it impossible to do such a comparison, particularly since other organic substances could have been leached, as well. Also, it was not possible to estimate the biomass specific rate of degradation of benzene at 21 °C and 4 °C. The problem is that the total aromatic compound concentration was less than that of protein, which was used to assess biomass. The protein concentration represents changes related to both degradation of benzene and of other organic compounds present as a result of the contamination (Tables 4.3-4.4, Figures 4.19-4.30).

The setup of this research was based on earlier research such as that by Lovely et al. (1994) and Lovely and Lonergan (1990). They did not report any release of contaminants from the rubber stoppers. Teflon-coated rubber stoppers were not a possible alternative to the butyl rubber, since oxygen can penetrate them as observed by Gribic'-Galic' and Vogel (1987). A solution is using serum bottles closed with Teflon-lined butyl rubber stoppers (Cozzarelli et al., 1994). Such bottles should have additional, outer seals to prevent penetration of oxygen. In addition, a gas-tight syringe should be used to collect the sample from the bottles, and only one sample sufficient for all analyses required should be drawn, as was done in this study.

Temperature affected the amount of contamination released from the butyl rubber stoppers. Almost double the concentration of ethylbenzene, p-xylene, and o-xylene was released at a temperature of 21 °C than at 4 °C. Benzene concentration did not change when other organic compounds were released in the serum bottles containing only autoclaved distilled water (Figure 4.33). The initial benzene concentration appeared to have no influence on the release of these aromatic compounds.

Although the contamination was unfortunate, it enabled us to observe the degradation of a range of aromatic hydrocarbons in addition to benzene. It is also important to note that nearly all benzene degradation took place early, while the contaminant concentrations were low. Hence, the other aromatics likely did not greatly influence the benzene degradation.

The other compounds were degraded possibly by different bacteria did not prevent benzene degradation. Since the ethylbenzene and xylenes did not prevent benzene degradation, the enzymes degrading benzene must have been substrate specific. Mutations modifying these enzymes might have occurred when the microorganisms were exposed to other aromatic compounds, new enzymes might have been induced or the new substrates could have resulted in growth of different bacterial species. Benzene continued to degrade at a slower rate after

ethylbenzene, p-xylene, and o-xylene were released, suggesting some competitive inhibition of benzene degradation or that a threshold benzene concentration was necessary for enzyme induction. Under sulfate-reducing conditions, p-xylene degraded first, followed by ethylbenzene and o-xylene, over a period of 190 days after the benzene was degraded (Figure 4.32). The much longer time to degrade the contaminant aromatics suggests that multiple groups of organisms were involved. The concentrations of xylenes were higher than that of ethylbenzene under iron-reducing conditions than under sulfate- reducing conditions at 21 °C.

The production of sulfide ions, and black precipitate, demonstrated the presence of sulfate reducers in the experimental bottles with sulfate containing medium. Ferrous ions added to the medium precipitated as FeS and this reaction kept sulfide concentration low and variable (Section 4.2.4.1). Sulfide was detected in some of the experimental bottles maintained 21 °C after at least 4 months (Figures 4.34-4.36). Also, beginning of the fourth month, black precipitate was detected in the 3 bottles that had been amended with 200 ppb benzene at 21 °C (Figure 4.36). There was no black precipitate or detection or odor of hydrogen sulfide at 4 °C, except after six months in the bottles that were amended with 200 ppb benzene. The controls did not have sulfide or black precipitate, which would exclude the chemical production of sulfide in this experiment. It appears that fermentation initiated biodegradation of

benzene and then the products were subjected to additional biodegradation by sulfate-reducing bacteria, the latter mostly in bottles at the highest temperature and with the greatest benzene concentration. Some values of sulfide concentration were greater than expected based on the stoichiometric amount that could be produced from the added benzene alone. This was probably due to the degradation of other aromatic compounds. The sulfate concentration was reported as 1.6 g/liter in almost all the cases because the test strip indicated ≥ 1.6 g/liter.

Highly crystalline hematite (Fe_2O_3) added to the medium for iron-reducing bacteria had low solubility. The rate of biological ferric iron reduction tends to decrease with an increasing degree of crystallinity. This indicates that the organisms must come into direct contact with Fe (III) in order to reduce it to Fe (II)(Munch and Ottow, 1983). Measuring the dissolved Fe (II) was not sufficient to measure the rate of Fe (III) reduction, since substantial Fe (II) precipitated in solid form as FeS.

Ferrous ions in solution were detected in the experimental bottles but not in the controls. The concentration of ferrous ions fluctuated over time since both production and precipitation were occurring. Chemical reduction should be considered, since iron can be reduced by sulfide. But the controls did not have Fe (II), excluding the chemical reduction of Fe (III) in this experiment.

The temperature and substrate concentration played a role in determining if benzene was biodegraded under conditions favorable to iron-reducing bacteria (Figures 4.6-4.8). Initially, the benzene degraded faster at 21 °C than at 4 °C in some bottles. The rate of benzene degradation slowed down when the concentration of benzene was less than 7 ppb. Ferrous iron was detected in the first month at 21 °C but not at 4 °C. At 21 °C the concentration of ferrous iron was greater than during the first month in the bottles that had been amended with 10 and 50 ppb than in the one that had been amended with 200 ppb benzene (Figures 4.40-4.42). Probably most of the ferrous iron detected during the first 2 months was the result of benzene degradation and that measured during the remaining 4 months was mainly due to degradation of the other compounds, ethylbenzene, p-xylene, and o-xylene. At 4 °C, ferrous iron was detected during the second month in the bottles that had been amended with 200 ppb benzene. Also, ferrous iron was detected during the third and fourth months in the bottles that had been amended with 10 and 50 ppb of benzene at 4 °C (Figures 4.43-4.45).

As was the case for sulfate containing bottles, the addition of Fe(III) did not increase the rate of benzene degradation. In a similar set of experiments, under conditions of methanogenesis, the degradation of benzene was also found. Additional details of these observations will be discussed in the following chapter. This suggests that fermenters rather

than iron-reducing bacteria probably initiated biodegradation of benzene. Since benzene degradation was more complete in the presence of Fe(III), iron reducing bacteria could have removed an inhibitory intermediate, as suggested for sulfate. Iron-reducing bacteria can metabolize fermentation products such as acetate and hydrogen (Lovely and Phillips 1987b; Balashova and Zavarzin, 1980).

4.5 Summary and Conclusions

The anaerobic microbial populations utilized in this study degraded benzene under conditions favorable to iron and sulfate reducing bacteria at both low and moderate temperatures. There was no apparent relationship between temperature or benzene concentration and the rate of biodegradation of benzene under conditions favorable to sulfate or iron-reducing bacteria, except at relatively low benzene concentrations. Benzene degradation was nearly complete in the first month; this is very rapid compared to earlier observations, probably because of the lengthy acclimation period. Phenol was detected as an intermediate.

The inadvertent addition of the other aromatics can be seen as having a positive aspect in that their degradation was also observed. These compounds were much more slowly degraded than benzene (Figure 4.32). This would not be expected *a priori* based on their structure; presumably this was due to a need to "acclimate" the microbial consortium to these substrates.

Most of the bacteria in the microcosms were gram negative rods, but the community was heterogeneous. At least some members of the bacterial populations could survive under extreme temperatures by forming spores as a defense mechanism.

The results of the laboratory experiments reported here are specific to well FMW 6894, east side of building 1060 in East QFS Area, Fort Wainwright, Alaska. These findings, however, will help to explain the biodegradation of benzene under iron- and sulfate- reducing conditions in similar field situations.

Practical application of the results in OU5 groundwater is possible. Analyses of this area done by University of Alaska Fairbanks and the U.S. Geological Survey, Fairbanks, Alaska (Appendix D) indicate *in situ* sulfate concentrations sufficient for sulfate reduction. The *in situ* biodegradation of benzene could be initiated if nutrients similar to those in the growth medium are added to OU5 groundwater.

CHAPTER FIVE

BENZENE DEGRADATION BY FERMENTATION AND METHANOGENESIS

5.1 Introduction

The groundwater in Operable Unit 5 (OU5) at Fort Wainwright, Alaska, has become contaminated with petroleum hydrocarbons. The most water-soluble components of the contaminants are the homocyclic aromatic compounds, benzene, toluene, ethylbenzene, and xylene (BTEX). Research is needed to study the degradation of benzene under anaerobic and low temperature conditions to better evaluate the potential contribution of anaerobic bioremediation to contaminated site restoration.

The research of Edwards and Gribic`-Galic` (1992, 1994), Evans et al. (1991), and Haag et al. (1991) indicates that bioremediation of groundwater contaminated with aromatic hydrocarbons, including benzene, is possible using mesophilic anaerobic bacteria. Vogel and Gribic`-Galic` (1986) showed that benzene was fermentatively oxidized with ^{18}O from ^{18}O-labeled water. Anaerobic biodegradation of aromatic hydrocarbons at temperatures below 10 $^{\circ}C$ has not been studied in detail previously.

Methanogens belong to the kingdom Archaebacteria. They are widespread in strictly anaerobic environments. Fermenting and methanogenic bacteria can establish a syntrophic association, but neither

of these groups, alone, can degrade complex organic compounds completely (Zender, 1978). Most methanogens can use only a limited range of substrates, such as H_2, CO_2, acetate and methyl groups of C-1 compounds (Fenchel and Finaly, 1995). Other fermenting bacteria must convert complex organic compounds such as aromatic hydrocarbons to H_2, CO_2, and acetate before the methanogenic bacteria can convert them to methane. The methanogens will cleave the acetate into CO_2 and CH_4 and use the H_2 produced by fermenters as the electron donor to reduce CO_2 to methane. These reactions are thermodynamically favorable under an extremely low partial pressure of H_2. The methanogens in this case drive the reaction forward by consuming H_2 (Strayer and Tiedje, 1978).

The primary goal of this chapter is to present information about the biodegradation of benzene and other BTEX compounds in groundwater, under methanogenic conditions and at low temperature. Understanding the transformation of benzene and other compounds could help to predict the fate of contamination in soil and groundwater in cold regions. More specifically, the following were objectives of this investigation: (1) to determine the degree of transformation and mineralization of benzene under anaerobic conditions and at temperatures of 4 $^{\circ}$C and 21 $^{\circ}$C; (2) to associate the activity of the microorganisms to the loss of benzene; (3) to understand the tolerance of the microorganisms for variations of environmental factors such as temperature and substrate concentration;

and (4) to characterize the groups of microorganisms involved in the biodegradation.

5.2 Materials and Methods

(See Sections 3.21-3.2.10)

5.3 Results

The medium used for this study was depleted of sulfate and ferric ions. This was confirmed using the methods discussed in Chapter Three for sulfate and ferric iron analysis.

In general, the benzene data presented in this chapter are the mean of three replicates. The benzene data for serum bottles analyzed monthly over a period of six months are in Appendix H. These data include the benzene concentrations, mean, standard deviation and the confidence limits for a small population calculated using the small sampling theory (Spiegel, 1994). The confidence coefficient (t_c) value was selected based on the confidence desired, in this case 90%.

Each replicate serum bottle developed independently over the 6 month incubation period. Hence some differences among replicate bottles were substantially greater than those expected due to analytical error. However, benzene concentrations of replicates were generally in close agreement; the mean standard duration of control replicates at 50 ppp was 6 ppb and the relative standard deviation averaged 11%. For the experimental active bottles nearly all concentrations measured were less

than 10 ppb. For the experimental active bottles the standard deviation for samples ≤ 10 ppb averaged 6 ppb. Larger differences among replicate bottles were observed at one month in some of the biologically active bottles. In these cases, 50 ppb benzene at 21 °C and 200 ppb benzene at 4 and 21 °C, some of the replicate bottles had decreased to very low concentrations, while benzene in some replicates remained higher. This indicates that there was considerable variability in the benzene degradation rate initially, but by two months benzene concentrations were ≤ 12 ppb in almost all biologically active replicates under all conditions.

The pH was 7 and remained constant during the period of incubation. The colorless resazurin indicator confirmed anaerobic conditions throughout the incubation.

5.3.1 Benzene Degradation

Figures 5.1-5.4 summarize the degradation of benzene under fermentative conditions. Figures 5.5-5.8 summarize the degradation of benzene in a control that contained mercuric chloride and in an autoclaved control. The initial benzene concentrations in the experimental active samples were 10, 50, and 200 ppb and in the controls, 50 ppb. The concentration of benzene in the head space, calculated using Henry's law, is summarized in Appendix I.

At all concentrations and temperatures tested, the benzene concentration decreased below 4 ppb within 5 months. Within a period of

six months, 60% of benzene was decomposed in the 10 ppb bottles at 21 °C and 100% in the 10 ppb bottles at 4 °C. The decrease in benzene was 92% in the 50 ppb bottles at 21 °C and 85% in the 50 ppb bottles at 4 °C. The benzene decomposition was 97% in the bottles with 200 ppb benzene at 21 °C and 96% in the 200 ppb bottles at 4 °C (Table 5.1, Figure 5.5).

During the first month, the concentration of benzene dropped rapidly, but the rate of decrease slowed after the second month when benzene concentration fell below 5-10 ppb. The apparent degradation rate over the first month was faster at 4 °C than at 21 °C in some of the bottles at all benzene concentrations. The trace concentration of residual benzene for all initial benzene concentrations at 4 °C and 21 °C was similar after six months, except in the bottles that had an initial benzene concentration of 10 ppb and were incubated at 4 °C, which had zero benzene after 4 months.

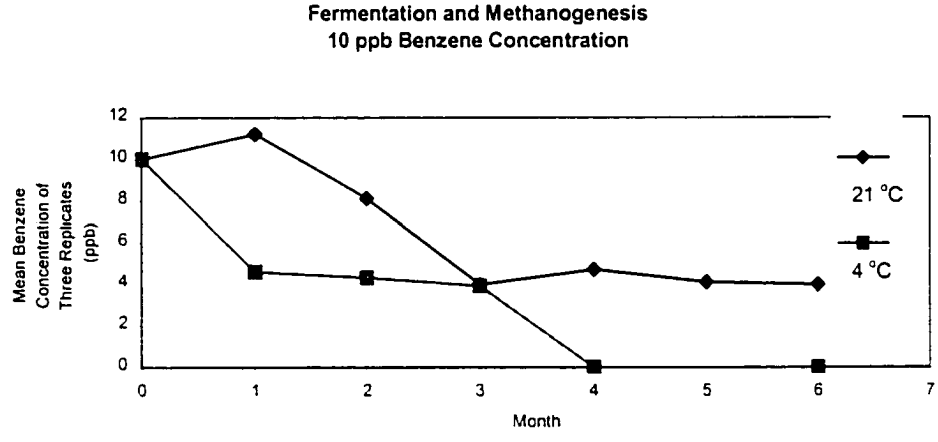

Figure 5.1: Biodegradation of 10 ppb of benzene over a period of six months at 21 °C and at 4 °C. The initial benzene concentration was calculated.

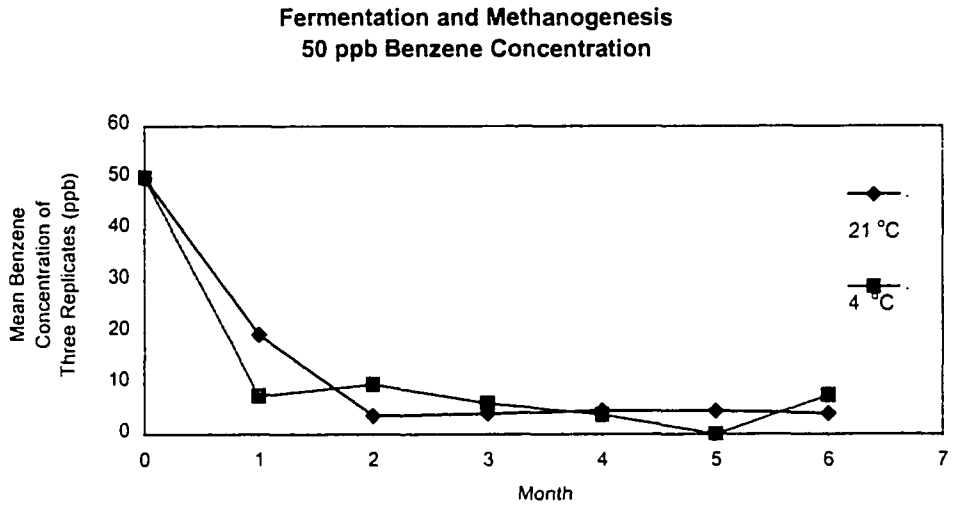

Figure 5.2: Biodegradation of 50 ppb of benzene over a period of six months at 21 °C and at 4 °C. The initial benzene concentration was calculated.

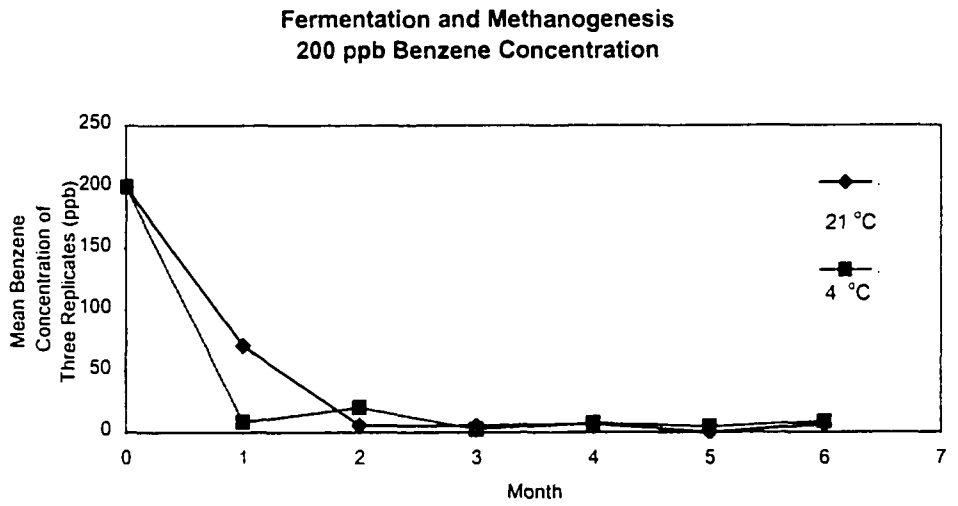

Figure 5.3: Biodegradation of 200 ppb of benzene over a period of six months at 21 °C and 4 °C. The initial benzene concentration was calculated..

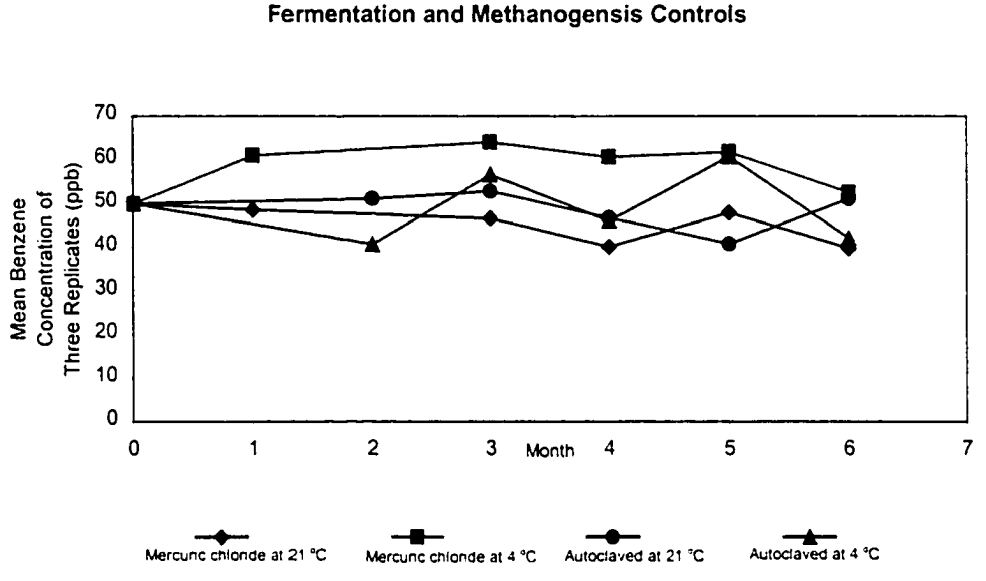

Figure 5.4: Degradation of 50 ppb of benzene in the control serum bottles over a period of six months at 21 °C and at 4 °C. The initial benzene concentration was calculated.

Table 5.1: Cumulative degradation (%) over a period of six months under conditions favorable to methanogenesis. The concentrations of benzene were the mean of the three replicates measured every month.

Fermentation and Methanogenesis			Fermentation and Methanogenesis		
Benzene 10 ppb at 21 °C			Benzene 10 ppb at 4 °C		
Month	Benzene (ppb)	Σ Degradation %	Month	Benzene (ppb)	Σ Degradation %
0	10.0	0	0	10.0	0
1	11.2	0	1	4.6	54
2	8.1	19	2	4.3	57
3	4	60	3	3.9	61
4	4.7	53	4	0.0	100
5	4.1	59	6	0.0	100
6	4	60			
Fermentation and Methanogenesis			**Fermentation and Methanogenesis**		
Benzene 50 ppb at 21 °C			**Benzene 50 ppb at 4 °C**		
Month	Benzene (ppb)	Σ Degradation %	Month	Benzene (ppb)	Σ Degradation %
0	50.0	0	0	50.0	0
1	19.5	61	1	7.5	85
2	3.7	93	2	9.8	81
3	4.1	92	3	6.1	88
4	4.6	91	4	3.8	92
5	4.6	91	6	7.6	85
6	4.1	92			
Fermentation and Methanogenesis			**Fermentation and Methanogenesis**		
Benzene 200 ppb at 21 °C			**Benzene 200 ppb at 4 °C**		
Month	Benzene (ppb)	Σ Degradation %	Month	Benzene (ppb)	Σ Degradation %
0	200.00	0	0	200.0	0
1	70.4	65	1	8.3	96
2	5.6	97	2	20.2	90
3	5.4	97	3	2.5	99
4	7.0	96	4	7.7	96
5	0.0	100	5	4.7	98
6	6.3	97	6	8.7	96

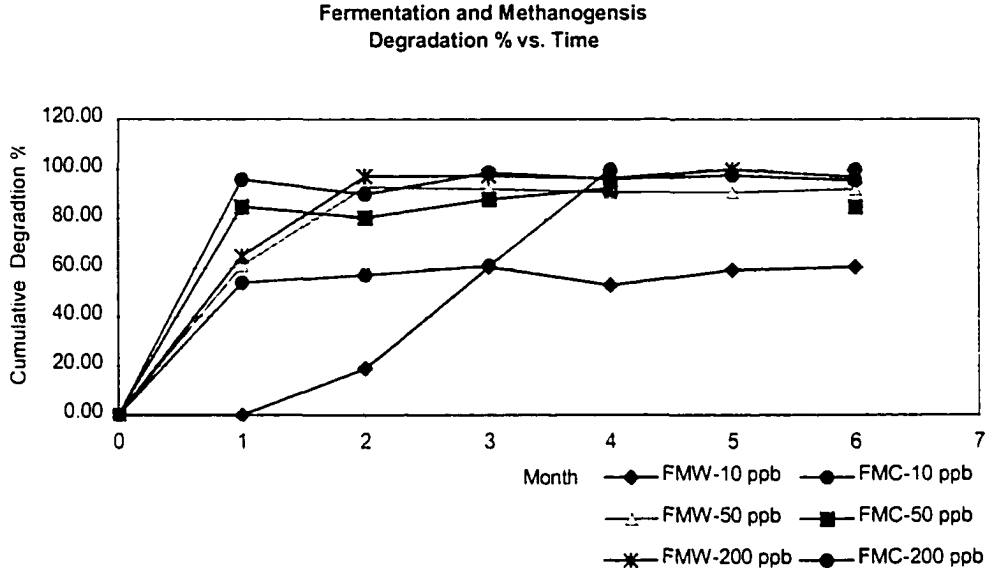

Figure 5.5: Cumulative degradation (%) over a period of six months under conditions favorable to methanogensis. The concentrations of benzene were the mean of the replicates measured every month.
FRW-10 ppb = Fermentation conditions, amended with 10 ppb benzene at 21 °C
FRC-10 ppb = Fermentation conditions, amended with 10 ppb benzene at 4 °C
FRW-50 ppb = Fermentation conditions, amended with 50 ppb benzene at 21 °C
FRC-50 ppb = Fermentation conditions, amended with 50 ppb benzene at 4 °C
FRW-200 ppb = Fermentation conditions, amended with 200 ppb benzene at 21 °C
FRC-200 ppb = Fermentation conditions, amended with 200 ppb benzene at 4 °C

5.3.2 Autoclaved and Mercuric Chloride Controls

In general, there was no decrease in benzene concentration in the autoclaved controls or controls with mercuric chloride at either temperature. Volatilization of benzene was negligible as indicated by Henry's law calculations (Appendix I).

5.3.3 Intermediates Detected During Benzene Degradation

The extraction method and GC/MS (See Chapter Three) were used to identify compounds present in selected incubation bottles that might be intermediates in benzene degradation. Phenol and benzoate were qualitatively detected during the analysis.

5.3.4 Contamination Released During Incubation

Ethylbenzene, p-xylene, and o-xylene were detected after the first month of incubation in both the experimental active and control bottles. As discussed in Section 4.2.3, the butyl rubber stoppers of the incubation bottles were the source of these compounds. The concentrations of such compounds increased over the six month period and were around two times greater at 21 °C than at 4 °C. The highest concentration was detected at the fifth month in the control bottles.

The p-xylene concentration was greater than those of ethylbenzene and *o*-xylene (Figures 5.6-5.9). Analyses were repeated seven months after the highest concentration was detected or one year

after the experiment started. Anaylses showed that the concentrations of these compounds had remained constant. No further measurements were done.

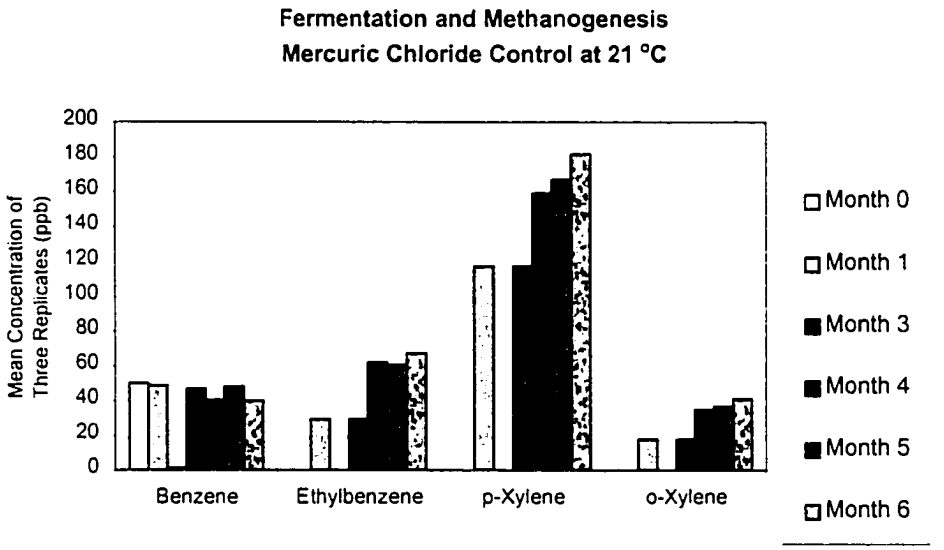

Figure 5.6: Mercuric chloride controls, amended with 50 ppb of benzene, over a period of six months at 21 °C. The interruption was due to no measurements being made. The initial benzene concentration was calculated

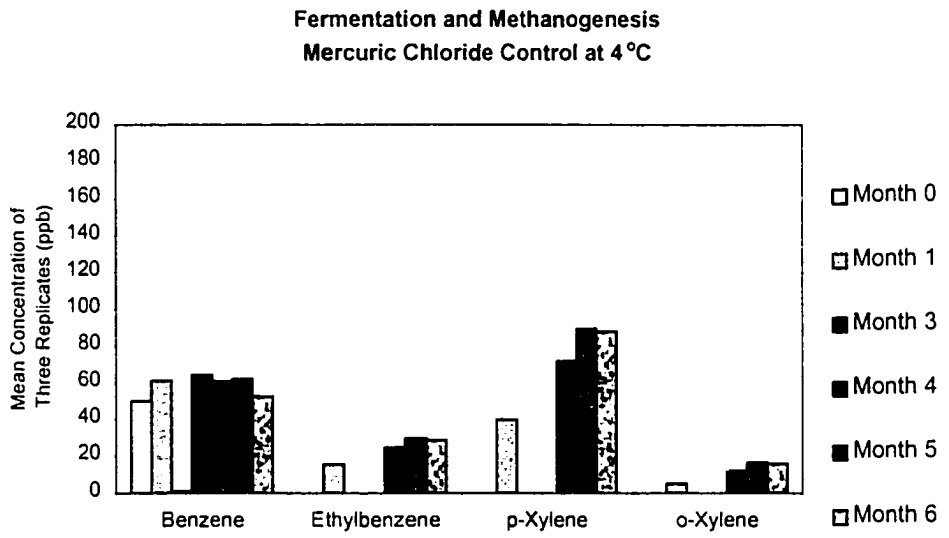

Figure 5.7: Mercuric chloride controls, amended with 50 ppb of benzene, over a period of six months at 4 °C. The interruption was due to no measurements being made for month two and for month three, excepting benzene. The initial benzene concentration was calculated.

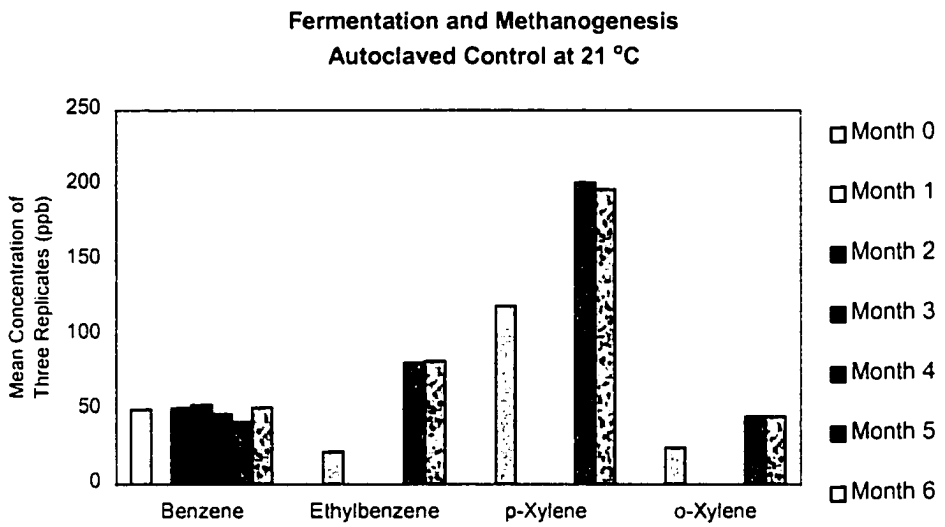

Figure 5.8: Autoclaved controls, amended with 50 ppb of benzene, over a period of six months at 21 °C. The interruption was due to no measurements at the indicated times. The initial benzene concentration was calculated.

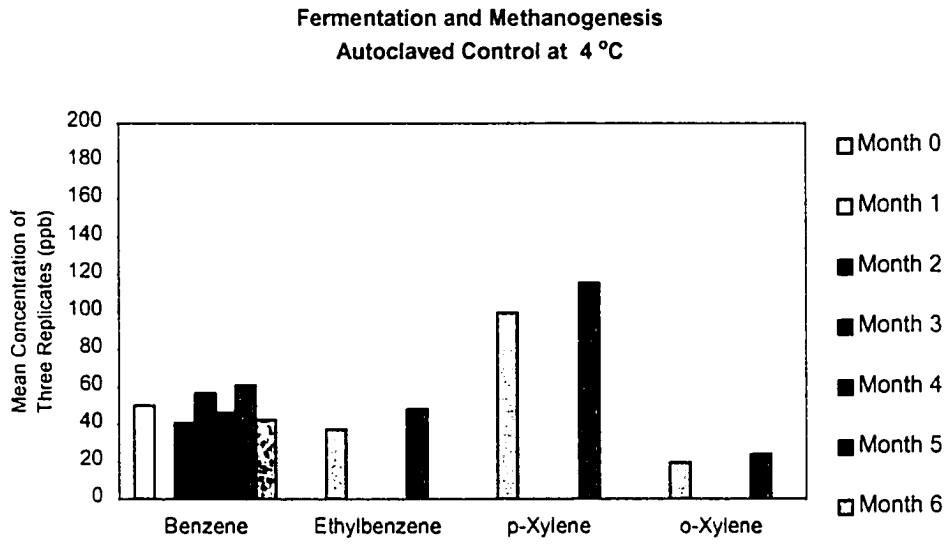

Figure 5.9: Autoclaved controls amended with 50 ppb of benzene over a period of six months at 4 °C. The interruption was due to no measurements at the indicated times. The initial benzene concentration was calculated.

As for the control bottles, the experimentally active bottles showed increases in ethylbenzene, p-xylene, and o-xylene. Also, the p-xylene concentration was greater than that of ethylbenzene or o-xylene. The concentrations of ethylbenzene and xylenes fluctuated during the first six months (Figures 5.10-5.15), but in general was less than or equal to those in the controls. After six months all combinations of experiments still had elevated ethylbenzene and xylene concentrations from 20-180 ppb. The fate of these compounds was followed over an additional period of 229 days at 4 °C. The concentration of benzene dropped from 8 to 5 ppb after 68 days and remained constant for the next 161 days. The concentration of p-xylene dropped from 150 ppb to 50 ppb at 229

days. The concentration of ethylbenzene decreased from 60 to 6 ppb after 172 days, then increased slightly to 22 ppb after an additional 57 days. The concentration of o -xylene dropped from 37 to 21 ppb in 172 days and increased again, up to 46 ppb, after an additional 57 days (Figure 5.16).

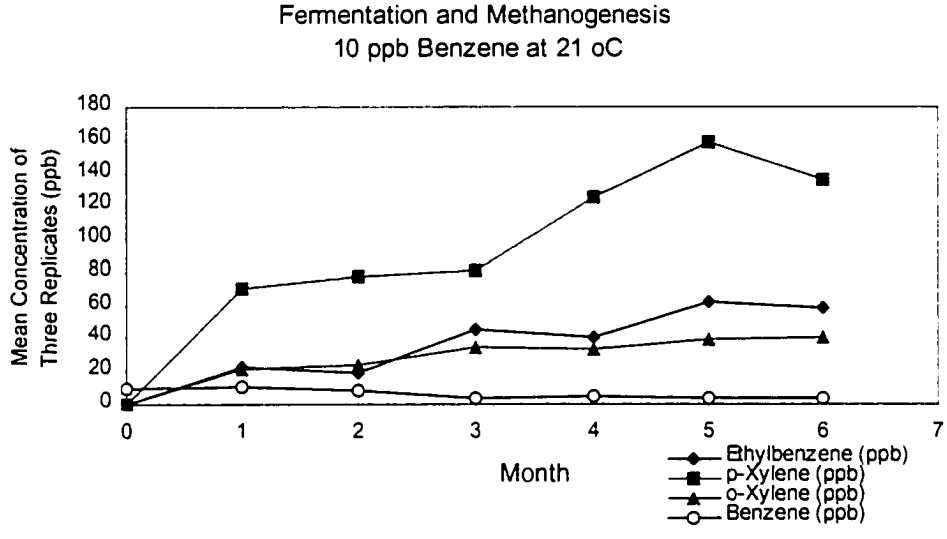

Figure 5.10: Biodegradation of 10 ppb of benzene under fermentation and methanogenesis conditions and the concentrations of contaminants over a period of six months at 21 °C. The initial benzene concentration was calculated.

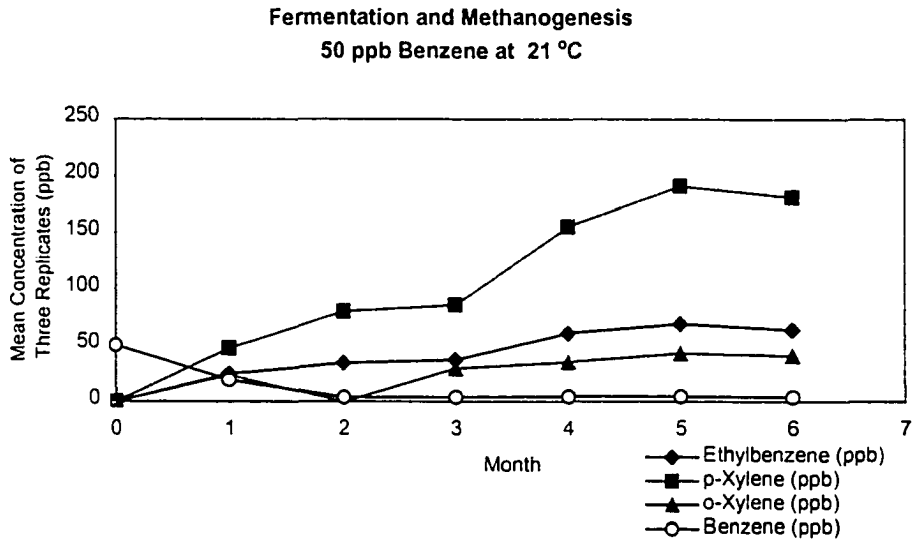

Figure 5.11: Biodegradation of 50 ppb of benzene under fermentation and methanogenesis conditions and the concentrations of contaminants over a period of six months at 21 °C. The initial benzene concentration was calculated.

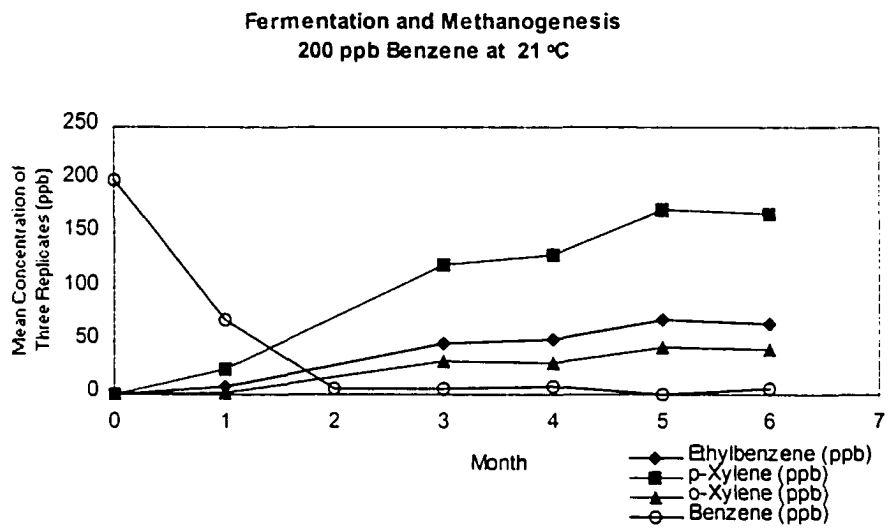

Figure 5.12: Biodegradation of 200 ppb of benzene under fermentation and methanogenesis conditions and the concentrations of contaminants over a period of six months at 21 °C. The initial benzene concentration was calculated.

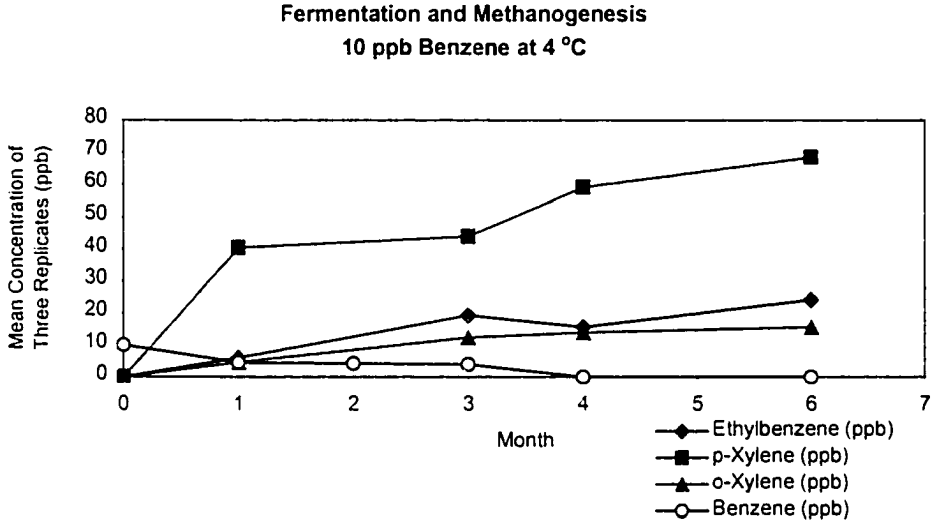

Figure 5.13: Biodegradation of 10 ppb of benzene under fermentation and methanogenesis conditions and the concentrations of contaminants over a period of six months at 4 °C. The initial benzene concentration was calculated.

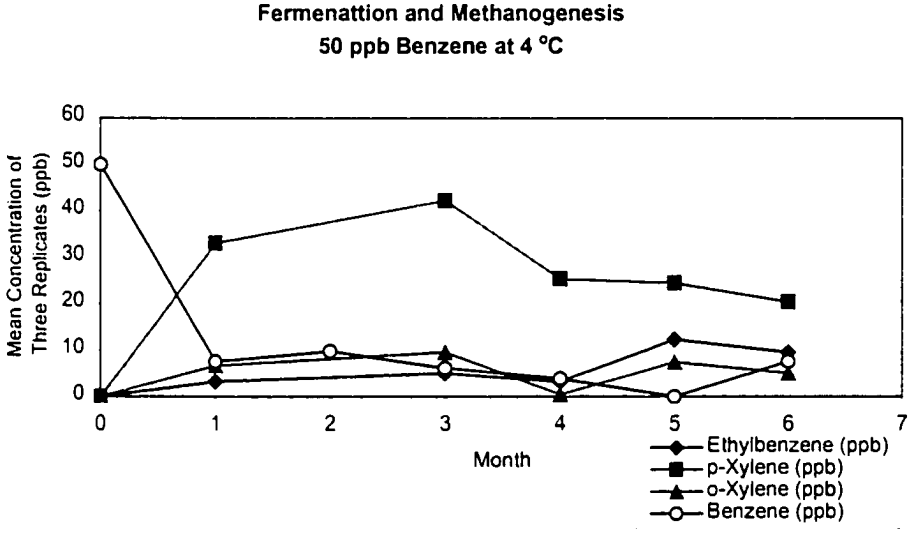

Figure 5.14: Biodegradation of 50 ppb of benzene under fermentation and methanogenesis conditions and the concentrations of contaminants over a period of six months at 4 °C. The initial benzene concentration was calculated.

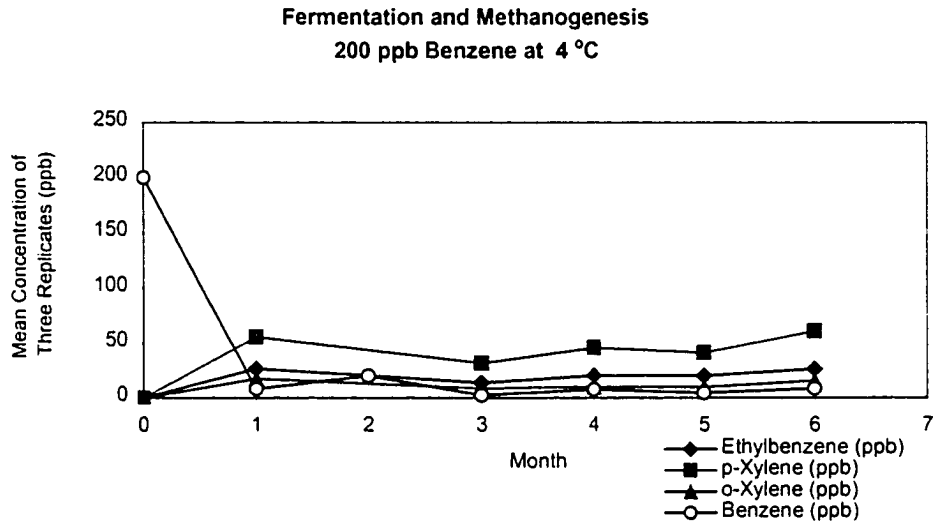

Figure 5.15: Biodegradation of 200 ppb of benzene under fermentation and methanogenesis conditions and the concentrations of contaminants over a period of six months at 4 °C. The initial benzene concentration was calculated.

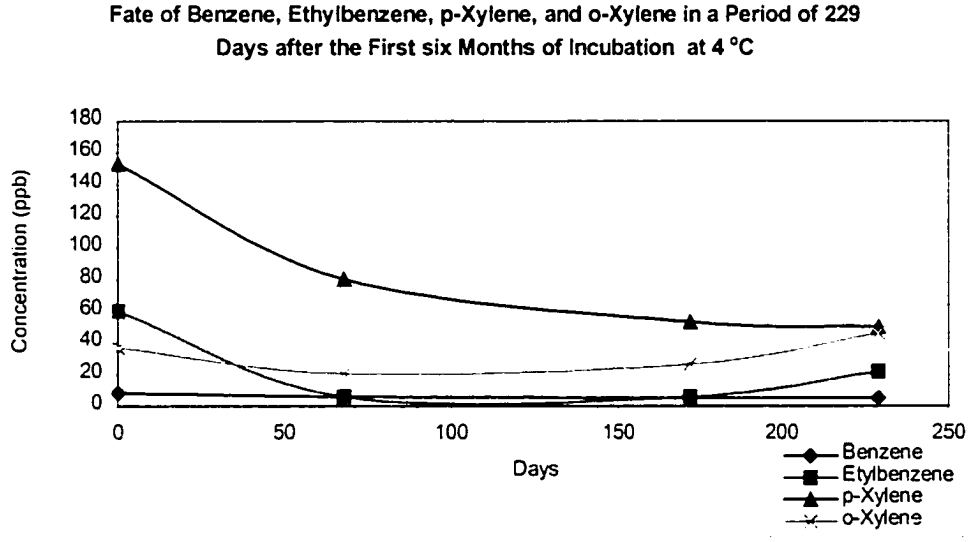

Figure 5.16: Degradation of benzene, and concentration of ethybenzene, p-xylene, and o-xylene under fermentation and methanogenesis conditions after the initial six month period.

5.3.5 Anaerobic Growth

A summary of protein concentration is shown in Table 5.2. Overall there was no trend in protein concentration with time. There was no change in color in the control serum bottles when tested for protein. The absorbance of the controls had a value approximately 1 on the protein standard curve. The 1 value was subtracted from the protein concentration values of the experimental bottles. Also, as mentioned before there were no bacterial cells detected under the microscope for the controls.

Table 5.2: The range of protein concentration over six months at 10, 50 and 200 ppb benzene and temperature of 21 °C and 4 °C. The protein was measured in (μg/ml).

Month	1	2	3	4	5	6
At 21 °C	Protein Conc.	Protein Conc.	Protein Conc.	Protein Conc.	Protein Conc.	Protein Conc.
Benzene Conc.						
10 ppb	1.4-2.5	0.2-0.4	1.5-1.7	0.8-2.7	2.1-4.8	0.4-2.5
50 ppb	1.4-2.5	0.2-0.4	1.5-1.7	0.8-2.7	2.1-4.8	0.4-2.5
200 ppb	0.4-0.7	0.1-0.5	0.1-1.3	0.8-2.7	0.7-2.5	1.2-2
At 4 °C	Protein Conc.	Protein Conc.	Protein Conc.	Protein Conc.	Protein Conc.	Protein Conc.
Benzene Conc.						
10 ppb	1.4-2.5	0.2-0.4	1.5-1.7	0.8-2.7	2.1-4.8	0.4-2.5
50 ppb	1.4-2.5	0.2-0.4	1.5-1.7	0.8-2.7	2.1-4.8	0.4-2.5
200 ppb	0.1-3.6	0.8-1.4	1.4-2.5	3-3.9	1.7-3.1	2.8-3.2

*Unit of Protein concentration is μg/ml

The consortium growing on benzene was heterogeneous. The shape of the cells was determined using light and epifluorescence microscopy and acridine orange stain. Rod-shaped cells (bacilli) were dominant over spherical cells (cocci) (Figure 5.17-5.18). The observed

cells were Gram-negative. After exposing the cells to severe temperatures of -20 °C and 80 °C for a period of one hour, microscopic observation confirmed the presence of capsules and spores. The organisms were not subjected to starvation, since benzene did not degrade completely.

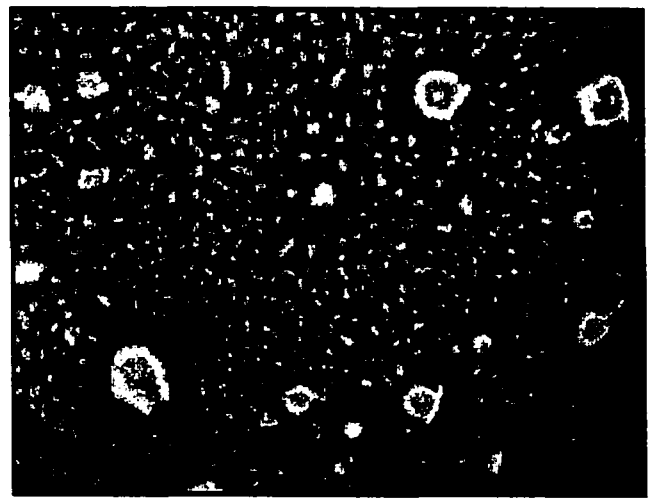

Figure 5.17: Rod-shaped cells (bacilli) were dominant over spherical cells (cocci) at under fermentation and methanogenesis conditions at 21 °C.

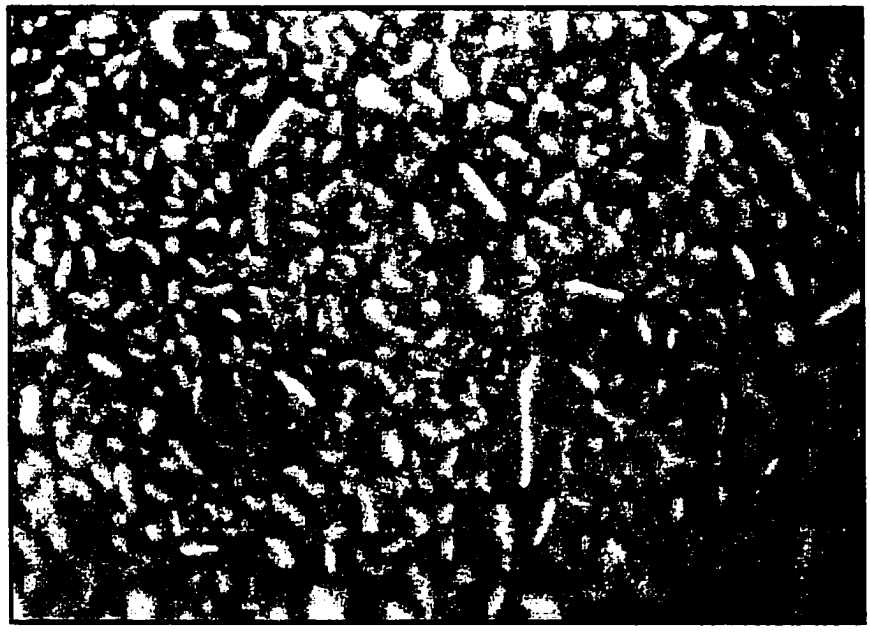

Figure 5.18: Rod-shaped cells (bacilli) were dominant over spherical cells (cocci) under fermentation and methanogenesis conditions at 4 °C.

5.3.6 Comparison of Experiments under Iron- and Sulfate-Reducing and Fermentation Conditions

In a similar set of experiments, under iron- or sulfate- reducing conditions (see Chapter Four), the degradation of benzene was also found. Overall there were no clear differences in benzene degradation rate under conditions favoring methanogenesis, compared with conditions favoring iron or sulfate reduction. The main difference was that benzene

degradation was usually less than 100% under conditions favoring fermentation and methanogenesis.

Table 5.3 summarizes the cumulative degradation (%) at the end of six months for the iron- and sulfate-reducing bacteria and fermentation and methanogenesis.

Table 5.3 Degradation (%) of benzene after six months incubation under conditions favorable for iron- and sulfate -reduction and fermentation and methanogensis. The values represent the mean benzene concentration of three replicates.

	Degradation (%) at 21 °C			Degradation (%) at 4 °C		
Benzene	IRW	SRW	FMW	IRC	SRC	FMC
10 ppb	100	57	60	100	100	100
50 ppb	100	92	100	100	100	85
200 ppb	98	97	98	98	94	96

SR = sulfate -reducing Bacteria
IR = iron-reducing bacteria
FM = fermentation and methanogenesis
W = 21 °C
C = 4 °C

Figure 5.19 summarizes the degradation (%) of benzene at several concentrations at both 21 °C and 4 °C.

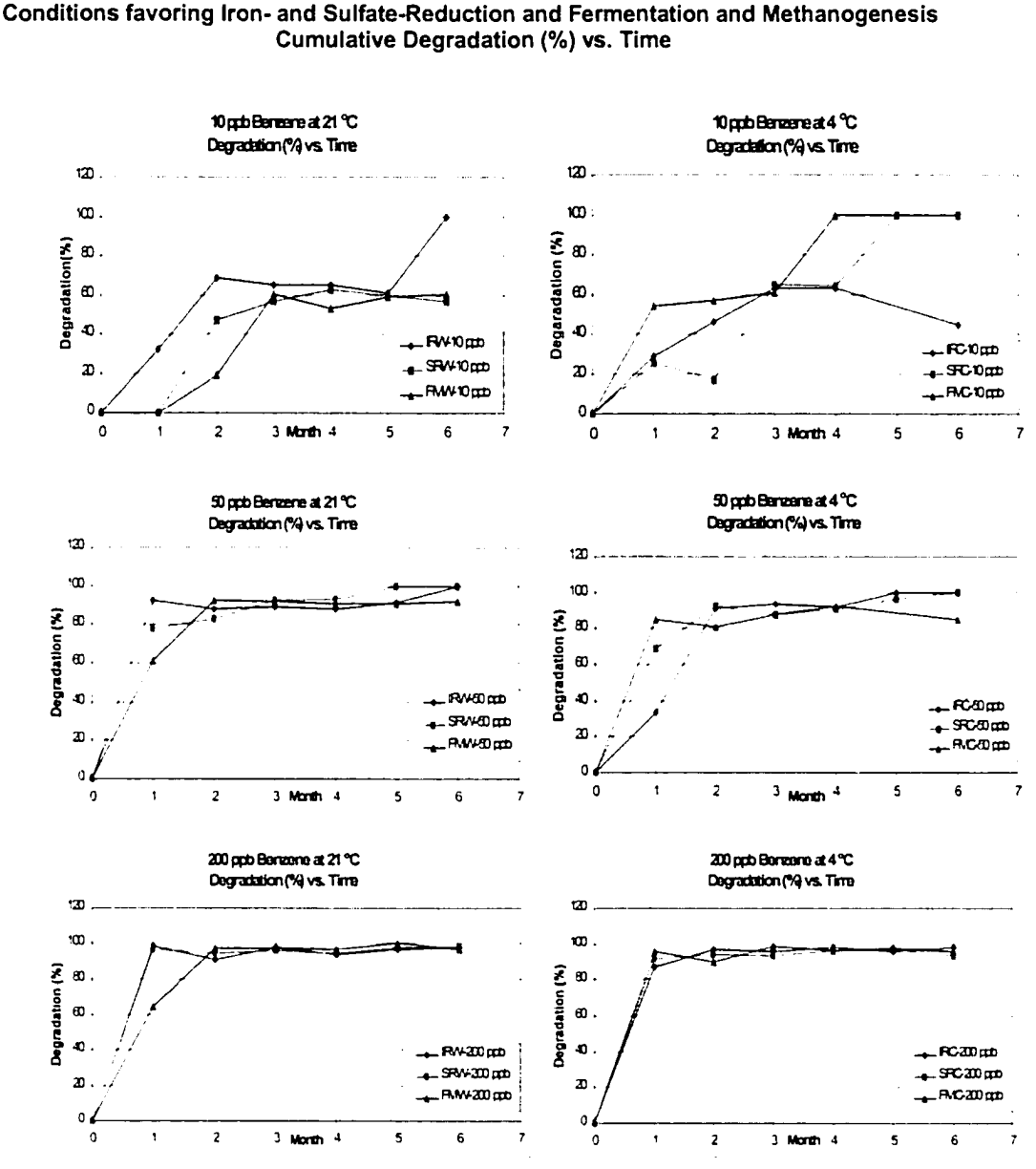

Figure 5.19: Degradation (%) of benzene over a period of six months under conditions favorable to iron- and sulfate -reducing bacteria and fermentation and methanogensis. The concentrations of benzene are the mean of three replicates measured every month.
SR = sulfate -reducing conditions
IR = iron-reducing conditions
FM = fermentation and methanogenesis
W = 21 °C and C = 4 °C

5.4 Discussion

The results of the study indicated that benzene had been anaerobically biodegraded both at 21 °C and 4 °C. For the control, only a 5% loss of benzene was observed. Chemical oxidation was thus eliminated as a possible cause of the loss of benzene, as were other processes such as adsorption to the stoppers or precipitates.

The reduction of benzene within a period of six months is summarized as the mean of three replicates (Table 5.2, and Figure 5.5, Appendix H). Except at 21 °C and 10 ppb initial benzene concentration, which had 60% degradation, nearly 100% of benzene was degraded within 6 months under all conditions. This is the first observation of anaerobic benzene degradation at low temperature in a system without sulfate or ferric iron.

The temperature affected benzene biodegradation under conditions favorable to methanogenic bacteria (See Section 5.2.1). Fermentation degraded benzene at both 21 °C and 4 °C. The biodegradation of benzene slowed down after the first month, at both 21 °C and 4 °C. This could have been due to the increasing concentration of the other aromatic compounds and competitive inhibition of benzene metabolism, or to the very low benzene concentration being below a threshold necessary for enzyme induction.

During the first two months, more replicate bottles for methanogenic conditions showed slow degradation at 21 °C (4 bottles)

than at 4 °C (1 bottle), which is contrary to the usual pattern of the rate being slower at low temperature (Figures 5.1-5.3). The microorganisms that initiated the transformation process could have had different acclimation periods at different temperatures. Also, the temperature played a role in the amount of contamination released from the butyl rubber stoppers. Almost double the concentration of ethylbenzene, p-xylene, and o-xylene were released at a temperature of 21 °C than at 4 °C. So, benzene might have degraded faster at 4 °C because of less inhibition by other substances.

The transformation pathway of benzene might include ring oxidation. In this research, GC/MS was used to detect, qualitatively, transient intermediates. A trace amount of phenol was detected in the biologically active benzene microcosms that was not detected in the controls. Similar findings were reported by Gribic`-Galic` and Vogel (1987) where phenol was detected as an intermediate during the transformation of benzene under methanogenic conditions. The biodegradation of phenol into gaseous products has been previously observed. Evans (1977), Gribic`-Galic` and Vogel (1987), and Lovely and Lonergan (1990) have all shown reaction pathways for the transformation of phenol under anaerobic conditions (Figures 2.3-2.5). Catechol, a typical intermediate for aerobic degradation of benzene, was not found.

The contaminants started degrading at a late stage, long after benzene had largely decomposed (Figures 5.10-5.15), apparently because there was a need for an acclimation period (Linkfield et al., 1989). Benzene was not degraded at a concentration of 5 ppb or less, in the presence of other aromatic compounds such as ethylbenzene, p-xylene, and o-xylene released from the stoppers. The concentration of the other contaminants, p-xylene, ethylbenzene, and o-xylene, fluctuated while the benzene concentration remained low and constant, perhaps because the stoppers were a continuing source. This is the first report of anaerobic degradation, under methanogenic conditions, for ethylbenzene, p-xylene, and o-xylene. There was no definite pattern for the rates of degradation of these contaminants.

In similar experiments, with iron (III) or sulfate added to the medium (Section 4.3), degradation of benzene also occurred. Biodegradation of benzene occurred at similar rates whether or not ferric iron or sulfate was present. Under all conditions, occasional replicates had elevated benzene concentrations, mainly at one month but a few at two months. There was no clear pattern in the occurrence of slower benzene degradation, either with temperature or with added electron acceptor.

In the case of iron- and sulfate-reducing bacteria, benzene degraded completely under most of the conditions, while a small residual concentration remained when sulfate or ferric iron was not present.

Addition of a low concentration of sulfate at 4 °C or ferric iron at 4 °C and 21 °C or made degradation more complete, consistent with the results of Gribic`-Galic` (1990).

It was impossible to estimate the biomass specific rate for degradation of benzene at 21 °C and at 4 °C. The problem we faced in this experiment was that the protein concentration needed to estimate the active biomass concentration over 6 months represents changes related to degradation of both benzene and other aromatic hydrocarbons present as a result of contamination by stoppers (Table 5.2).

The detection of protein of the total cell count confirmed the presence of bacteria in the experimental (non- control) bottles. There was no detection of bacterial cells in the controls. The microbial population was diverse and heterogeneous. Filamentous methanogenic bacteria were dominant in the experimental active bottles, differing from the rod shaped bacteria in microcosms with added sulfate or ferric iron (Figures 4.49-4.50, and Figures 5.18-5.19). Some of these microrganisms might adapt to a low-nutrient environment (Hirsch and Rades-Rohkohl, 1983; Godsy and Goerlitz, 1978) or to fluctuating oxidation-reduction conditions by forming spores, as in the natural environment (Gribic`-Galic`, 1990).

5.5 Summary and Conclusions

Fermentative microorganisms incubated under conditions favorable to methanogenesis biodegraded benzene. Such anaerobic microbial

populations degraded benzene at both 21 °C and at 4 °C. Availability of other aromatic substrates might have been a factor in slowing the degradation of benzene. The metabolic pathway used by methanogenic cultures was suggested by the presence of phenol in these experiments.

In situ biodegradation is a potentially economical process to clean up contaminated anaerobic aquifers and groundwater. Minimal additions of nutrients would be required, and no aeration is needed. In the case of fermentation and methanogenesis, there is no need to add an exogenous electron acceptor.

Future research is needed to better understand *in situ* biodegradation under methanogenic conditions and its rate in natural environments in similar field situations. The importance of biodegradation compared to other processes, such as dispersion, sorption, and volatilization, needs to be evaluated.

CHAPTER SIX

SUMMARY

The major hypothesis of this dissertation was that indigenous microorganisms from wells that have been contaminated for a long period of time with BTEX can acclimate, *in vitro*, to using benzene as a substrate under conditions favoring iron- and sulfate-reduction, and methanogenesis. The results of this research were consistent with this hypothesis. Benzene was substantially biodegraded within one to two months under anaerobic conditions at both 4 °C and 21 °C in the presence of several electron acceptors. This thesis includes the first report of anaerobic benzene degradation at a temperature as low as 4 °C. The *in vitro* research conducted on anaerobic biodegradation of benzene provided new insight into *in vitro* and *in situ* anaerobic degradation processes at the cold temperatures associated with sub-arctic environments.

Because other aromatic hydrocarbons were leached into the incubation vessels from the rubber stoppers used, it was also found that ethylbenzene, o-xylene, and p-xylene could be anaerobically biodegraded under the same conditions. These compounds degraded much more slowly than benzene, probably because the microorgansims were not previously acclimated to them.

The anaerobic transformation of benzene appears to include ring oxidation. Phenol was found to be an aromatic intermediate, and intermediates characteristic of aerobic degradation such as catechol were not found. Anaerobic biodegradation of hydrocarbons could result in the accumulation of significant low-molecular-weight organic acids in groundwater that were not present initially.

After initially rapid decomposition to concentrations less than 10 ppb, benzene continued to degrade at a much lower rate in the presence ethylbenzene, o-xylene, and p-xylene. This may have been due to some degree of competitive inhibition. Alternatively, a threshold concentration of benzene could be needed for enzyme induction. In the case of iron- and sulfate-reducing bacteria, benzene degraded completely under most of the conditions, but there usually was a small residual benzene concentration, less than 10 ppb, when these electrons acceptors were not added. Benzene degradation was more complete when sulfate and ferric iron were added to the media.

The presence of sulfate and ferric iron did not affect initial rate of benzene degradation. This is probably because a fermentation pathway that does not involve these electron acceptors initially degrades benzene.

The temperature played a minor role in determining the rate at which benzene was transformed. At 21 °C the biodegradation of benzene was faster in some replicates when ferric iron was available as an electron

acceptor 4 °C. However there was no clear temperature difference when sulfate was the electron acceptor, and under conditions favoring methanogenesis, benzene degradation was more often slower at 21 °C than 4 °C .

The microorganisms were heterogeneous, ranging from short rods and cocci to filaments. The filamentous bacteria were more numerous under conditions without added sulfate. Some microorganisms that were exposed to extreme temperatures formed spores. There is a possibility that some of these microorganisms were spore-forming fermenters and adapted to a low-nutrient environment (Hirsch and Rades-Rohkohl; 1983). In general, a few anaerobic bacteria can adapt to different electron acceptors.

In situ biodegradation is potentially an alternate economical process to clean up contaminated aquifers and groundwater, since minimal nutrient addition is required, no aeration is needed, and less biomass is produced. Especially favorable is the case of fermentation and methanogenesis, where there is no a need to add an exogenous electron acceptor.

The results of the experiments reported here are specific to the well FMW 6894, on the east side of building 1060 in East QFS Area, Fort Wainwright, Alaska. The benzene concentration in that well was constant over a period of four years and is believed to have remained constant

since its construction in 1994. The groundwater was analyzed over that period, and there was evidence for the existence of many other organic chemicals. These chemicals are structurally easier to use as substrates than benzene. This might explain why the benzene concentration has not changed. However, Kazumi et al. (1997) inoculated benzene-oxidizing microorganisms derived from other aquatic sediments into an inactive aquifer sediment, and rapid oxidation was noted (Kazumi et al., 1997). This gives us hope that the benzene-oxidizing microorganisms enriched in our laboratory, if inoculated into groundwater with the appropriate nutrients, are a possible solution to the remediation of contaminated groundwater. Microorganisms might play a role in determining the degradation rate of benzene *in situ*, which might be facilitated by the presence of other substrates in the natural environment. Future research is needed to determine the specific organisms responsible for Fe (III) and SO_4^{-2} reduction and methanogenesis of benzene- contaminated groundwater and their growth rates in natural groundwater systems. In addition, research is needed to achieve the practical application of in situ biodegradation of benzene in a sub-arctic environment.

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APPENDICES

APPENDIX A

OPERABLE UNIT 5 (OU5), EAST SECTION, FORMER QUARTERMASTER'S FUELING SOURCE AREA (EAST QFS AREA), FORT WAINWRIGHT, ALASKA

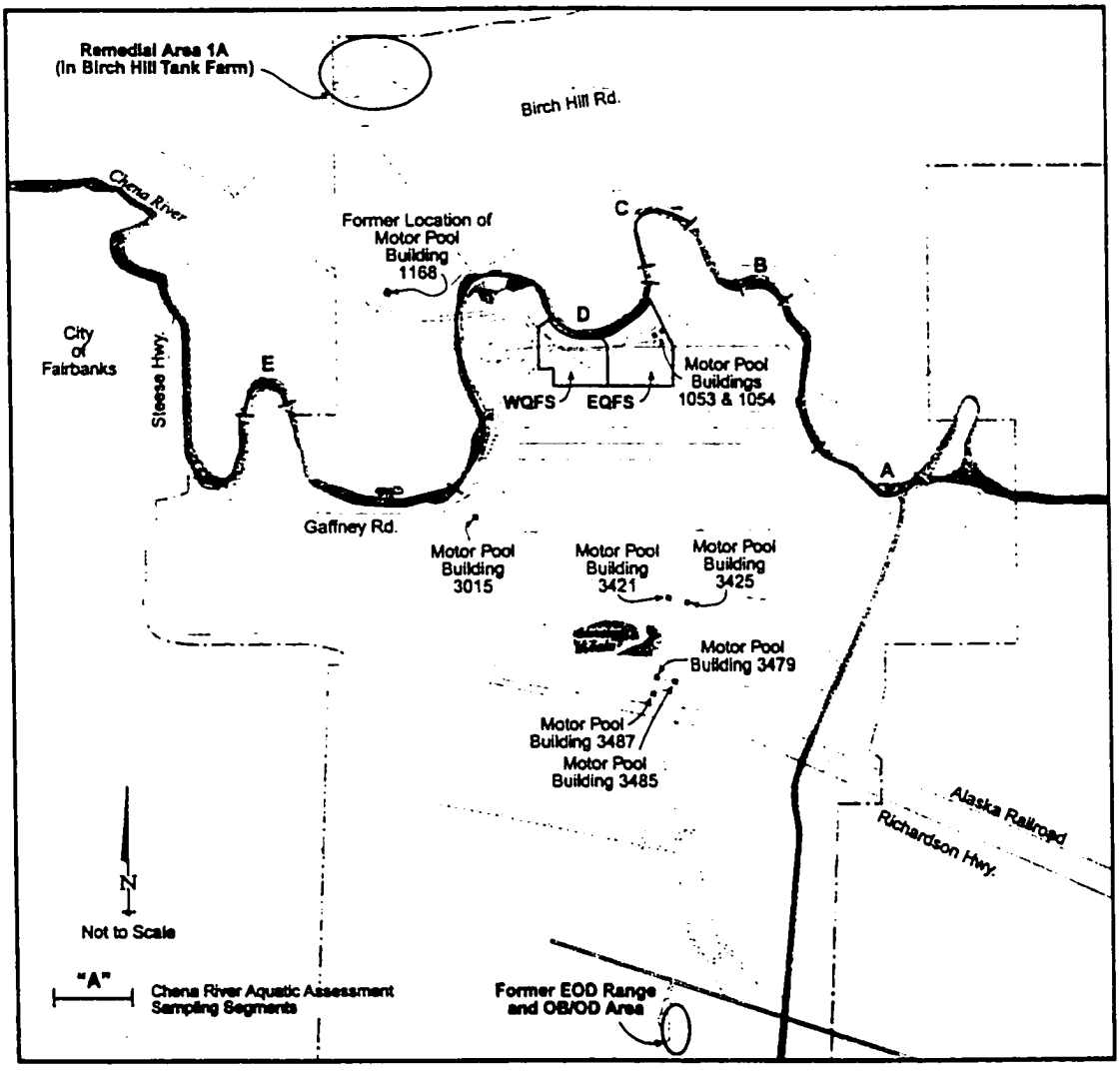


Figure A1: Fort Wainwright Site Map with OU5 Areas

* Source: Proposed plan for remedial action at Operable Unit 5, Fort Wainwright, Alaska June, 1998.

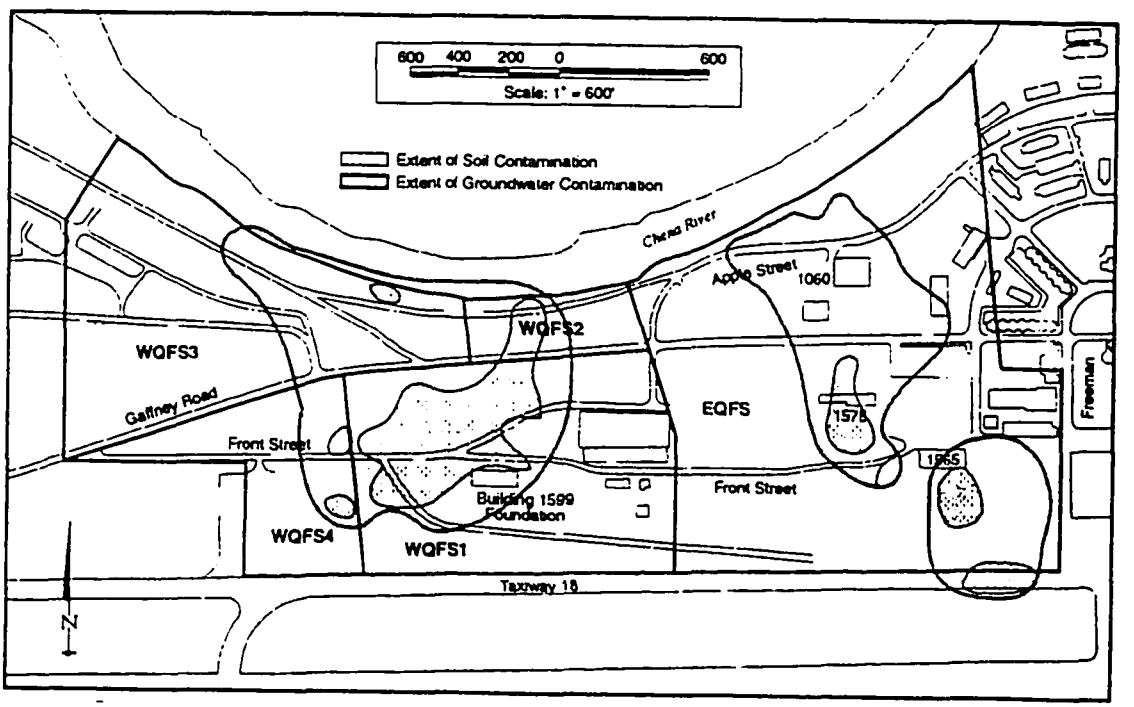


Figure A2: Extent Of Groundwater and Soil Contamination OU5

*Source: Record of Decision for Operable Unit 5 (OU5), Fort Wainwright, Fairbanks, Alaska, May, 1999.

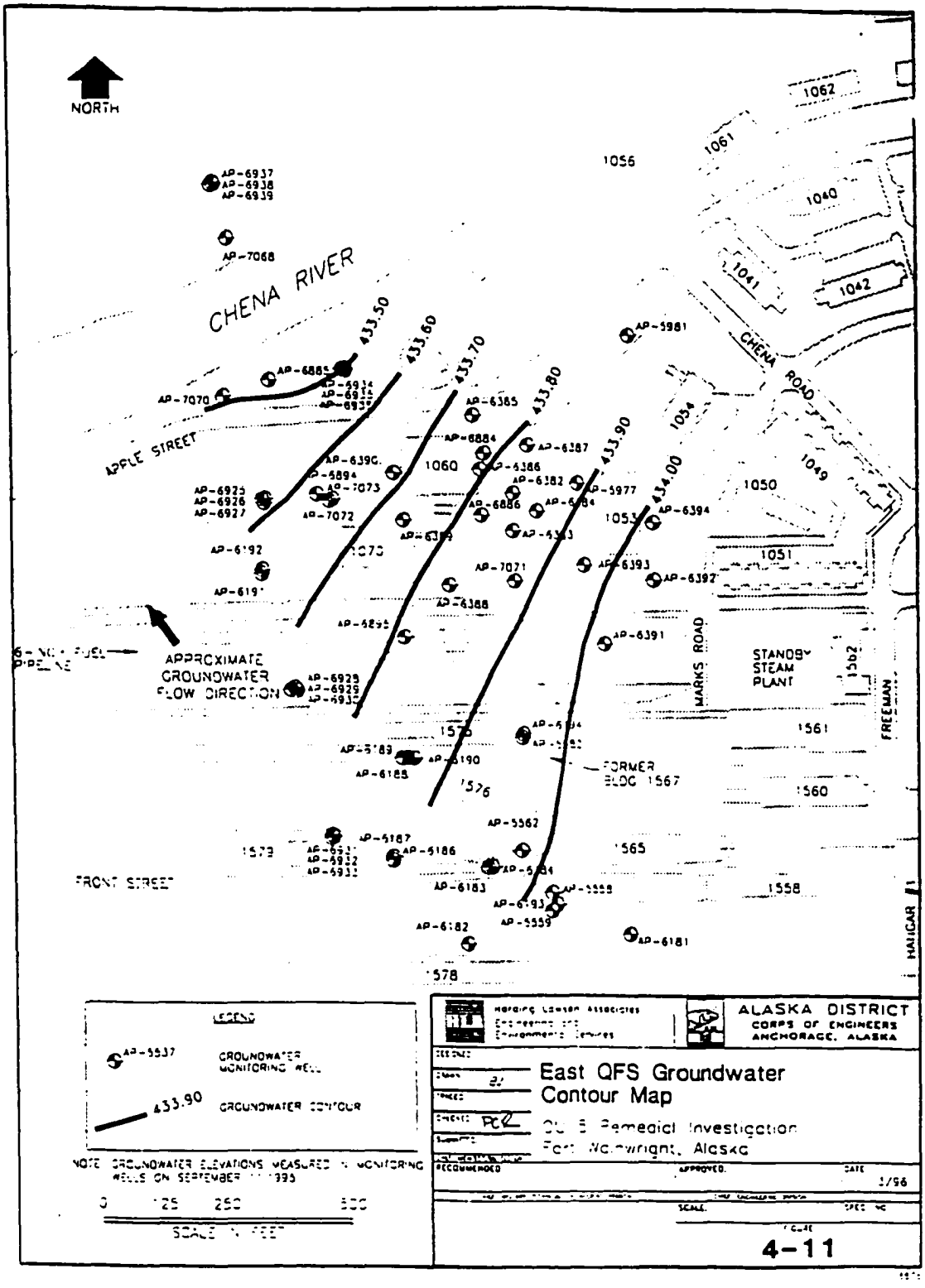


Figure A3: Location of well AP 6894 (FMW 6894) at Operabe Unit 5 (OU5), east section, Fort Wainwright, Alaska.

APPENDIX B

GENERALIZED SUBSURFACE PROFILE, EAST QFS GEOLOGIC CROSS-SECTION G-G'

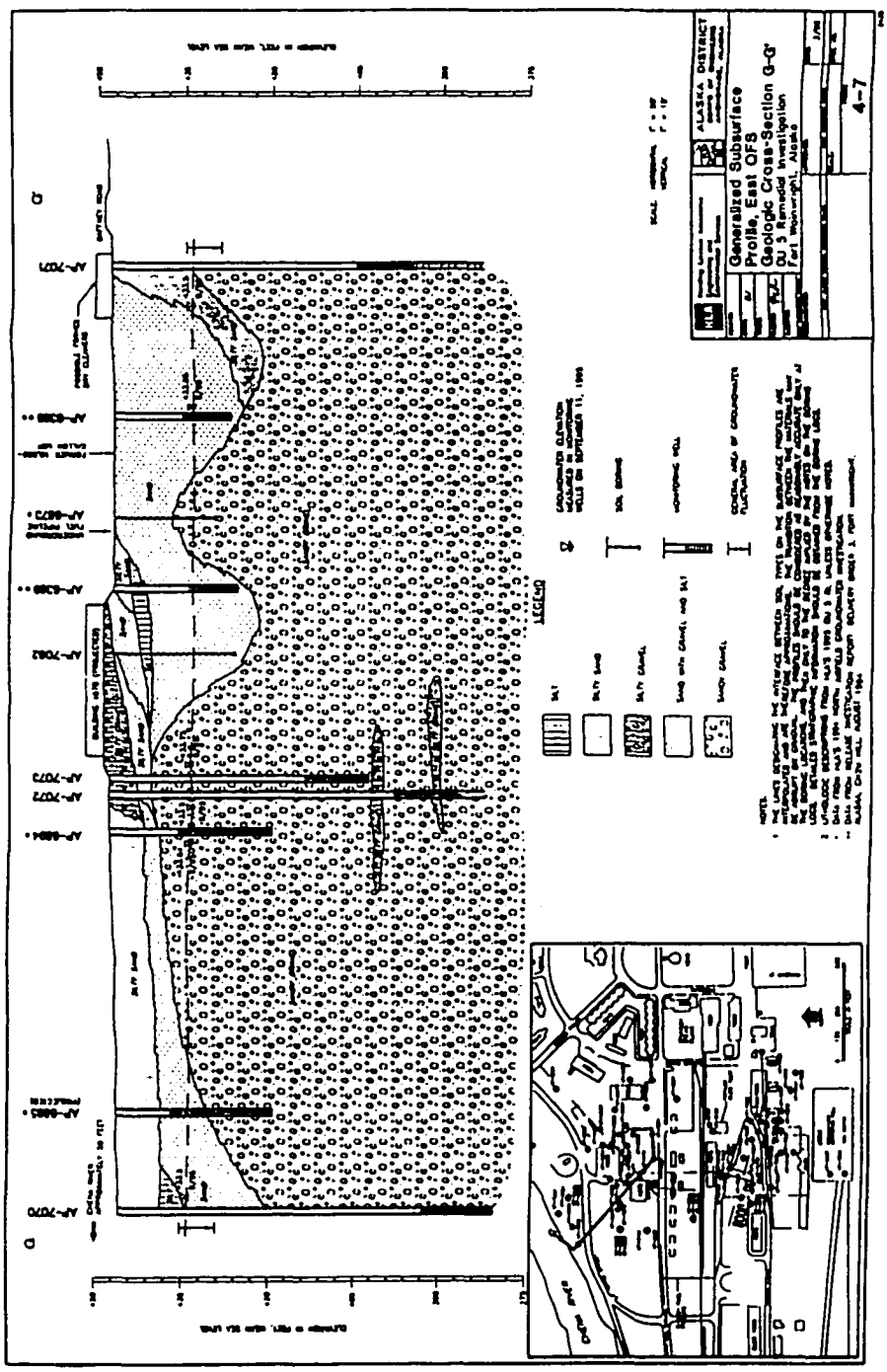


Figure B1: Generalized subsurface profile, east QFS geologic cross-section G-G', Fort Wainwright, Alaska

APPENDIX C

HISTORICAL AERIAL PHOTOGRAPHS

CONTENTS

FIGURES

Figure C1: September 9, 1949, Aerial photograph, West and East QFS Areas

Figure C2: July 17, 1962, Aerial photograph, West and East QFS Areas

Figure C3: August 10, 1969, Aerial Photograph, West QFS Areas

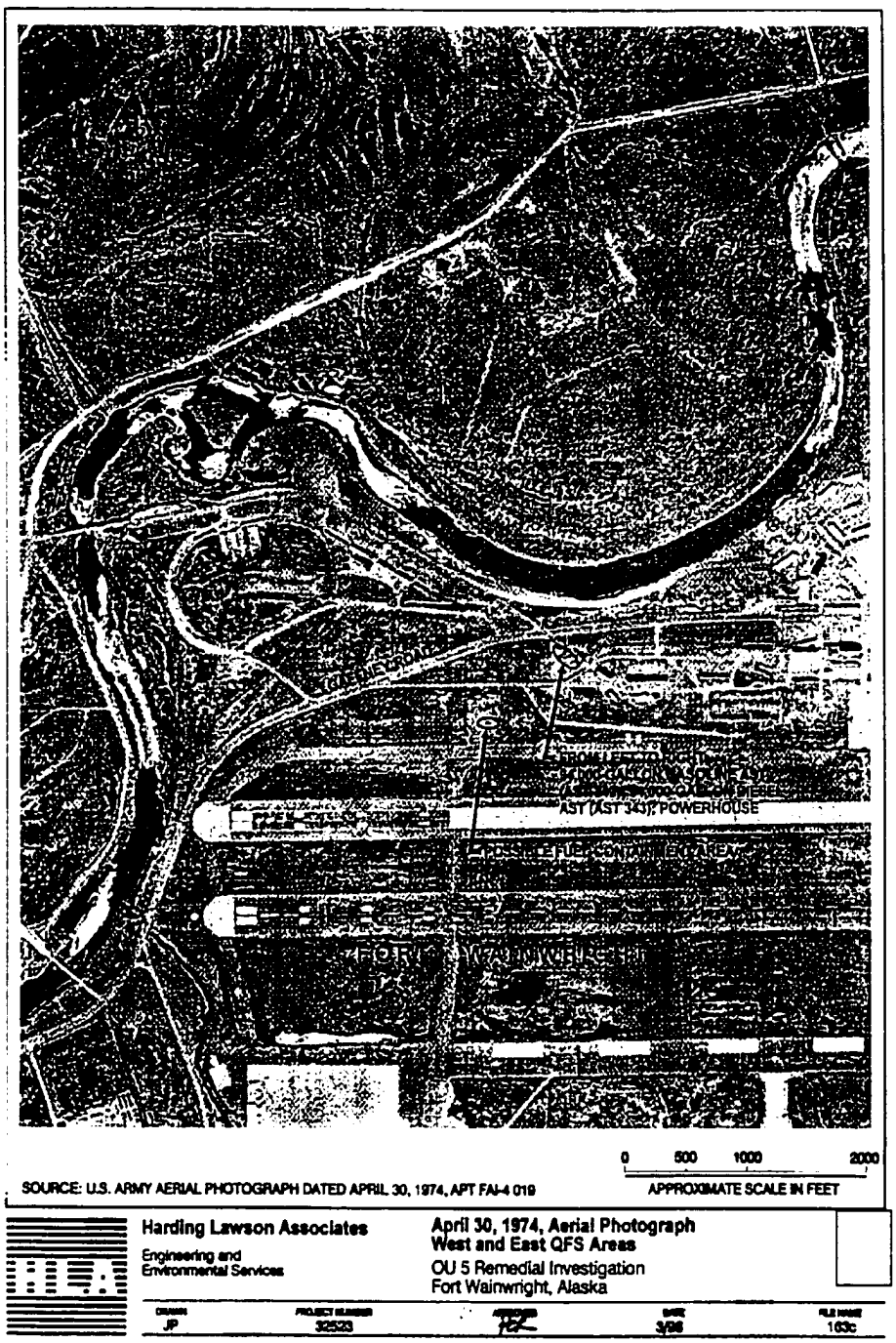


Figure C4: April 30, 1974, Aerial photograph, West and East QFS Areas

Figure C5: September 23, 1977, Aerial photograph, West QFS

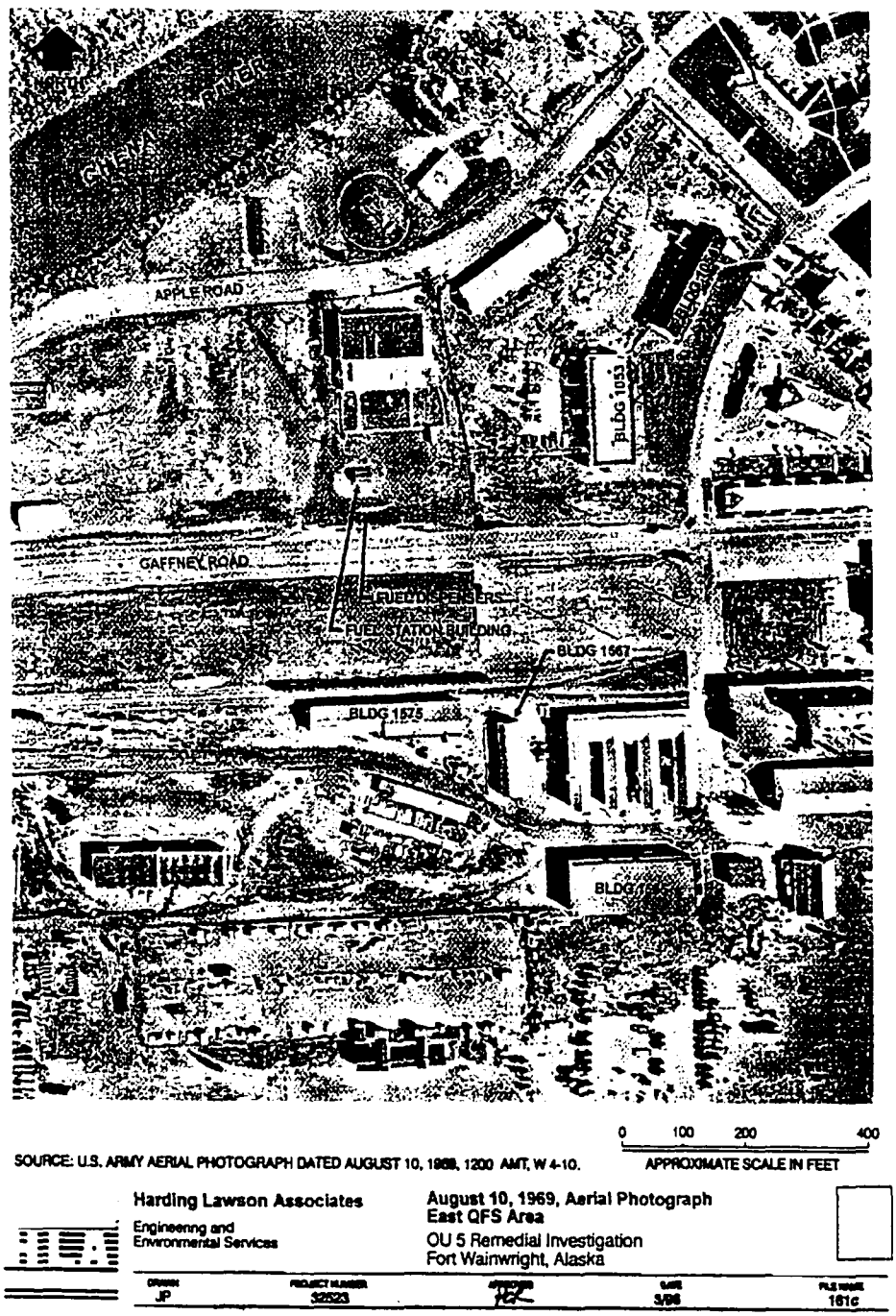


Figure C6: August 10, 1969, Aerial photograph, East QFS Areas

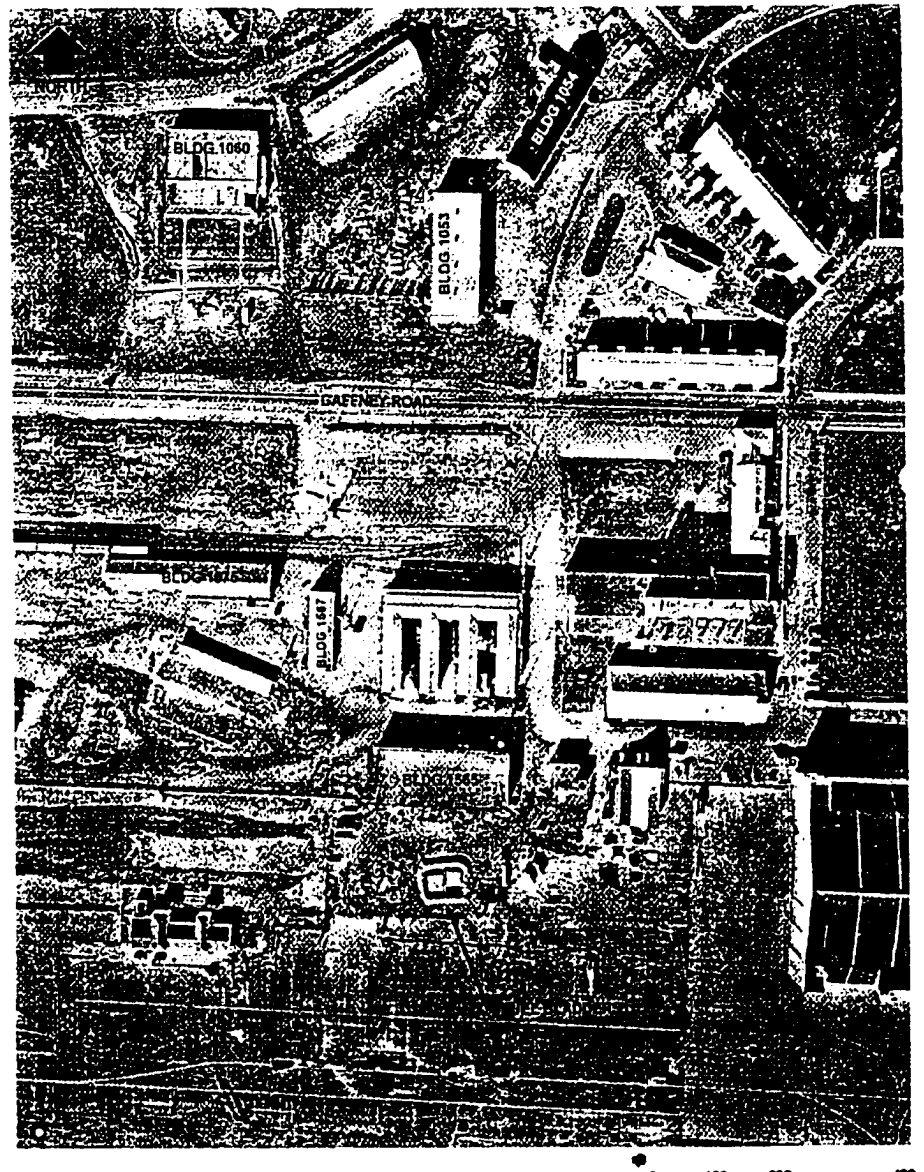


Figure C7: September 23, 1977, Aerial photograph, East QFS Areas

APPENDIX D

DATA COLLECTED BY UNITED STATES GEOLOGICAL SURVEY AND THE WATER AND ENVIRONMENTAL RESEARCH CENTER, UNIVERSITY OF ALASKA FAIRBANKS

Table D1: Data collected by USGS and Water and Environmental Research Center, University of Alaska Fairbanks.

	Well	FWM 6885	FWM 6894	FWM 7070	FWM 7072	FWM 7073	FWM 7075
Analyte	Units	9/19/96	9/19/96	9/19/96	9/19/96	9/19/96	9/19/96
NH3	mg/L	0.05	0.14	0.06	0.1	0.07	0.11
TKN	mg/L	-	-	-	-	-	-
NO2	mg/L	0.01	0.01	0.01	0.01	0.01	0.01
NO3	mg/L	0.08	0.07	0.06	0.08	0.06	0.06
NO2+NO3	mg/L	0.09	0.08	0.07	0.09	0.07	0.07
TOC	mg/L			2.8		3.2	
P	mg/L	0.01	0.02	0.04	0.01	0.02	0.05
SO4	mg/L	23	15	23	23	24	21
H2	nM	0.9	0.52		NA	0.19	
CH4	ppm	6.31	8.27	2.36	2.94	2.23	0.21
HC degraders	MPN/ml						0
Gas degraders	cells/ml	0	41	0	7.15	71.7	28.6
Diesel degraders	cells/ml	1.65	32.4	1.65	289	28.1	269
Toluene degraders	cells/ml	0	0	80	607	21.8	0
Total Hetero	cfu/ml	2280	47100	172	286000	48900	196000

NA = not analyzed

APPENDIX E

BACTERIAL GROWTH MEDIA

BACTERIAL GROWTH MEDIA

Content of the Bacterial Growth Medium

The modified medium consists of the following chemicals added to one liter of deionized water:

0.1 g $MgCl_2.H_2O$

0.6 g $NaH_2PO_4.H_2O$

10 ml phosphate buffer (27 g/l KH_2PO_4, 24.8 g/l K_2HPO_4)

10 ml of salt solution (53.5 g/l NH_4Cl, 7 g/l $CaCl_2.6H_2O$)

2 ml of trace mineral solution (see below)

1 ml redox indicator stock solution (1 g/l resazurin)

100 mg L-cysteine HCl

10 ml saturated bicarbonate solution (260 g/l $NaHCO_3$)

10 ml filter-sterilized vitamin stock solution

2 g $FeCl_2.4H_2O$ (for sulfate-reducing bacteria only)

3.55 g Na_2SO_4 (for sulfate-reducing bacteria only)

0.1 g sodium sulfide hydrate (for iron-reducing and methanogenic bacteria only)

0.05 g Fe_2O_3, hematite (for iron-reducing bacteria only)

0.1 g $FeCl_2.4H_2O$ (for methanogenic bacteria ony)

The vitamins and bicarbonate solutions were added from sterile stock solutions after autoclaving and cooling the medium and while gassing the medium with N_2/CO_2 (80%/20%). The pH was adjusted to 7 by adding 1 N NaOH or 1 N HCl.

Trace Minerals Added to Medium

A stock solution of trace minerals was prepared and stored at 4 oC. It consisted of the following:

0.3 g/l H_3BO_3

0.1 g/l $ZnCl_2$

0.75 g/l $NiCl_2.6H_2O$

1 g/l $MnCl_2.4H_2O$

0.1 g/l $CuCl_2.2H_2O$

1.5 g/l $CoCl_2.2H_2O$

0.02 g/l Na_2SeO_3

0.1 g/l $AlCl_3$

0.01 g/l $NaMoO_4.2H_2O$

Vitamins Added to Medium

A stock solution of vitamins was prepared and stored at 4 °C. It consisted of the following:

0.02 g/l biotin

0.02 g/l folic acid

0.1 g/l pyridoxine hydrochloride

0.05 g/l riboflavin

0.05 g/l thiamine

0.05 g/l nicotinic acid

0.05 g/l pantothenic acid

0.05 g/l PABA

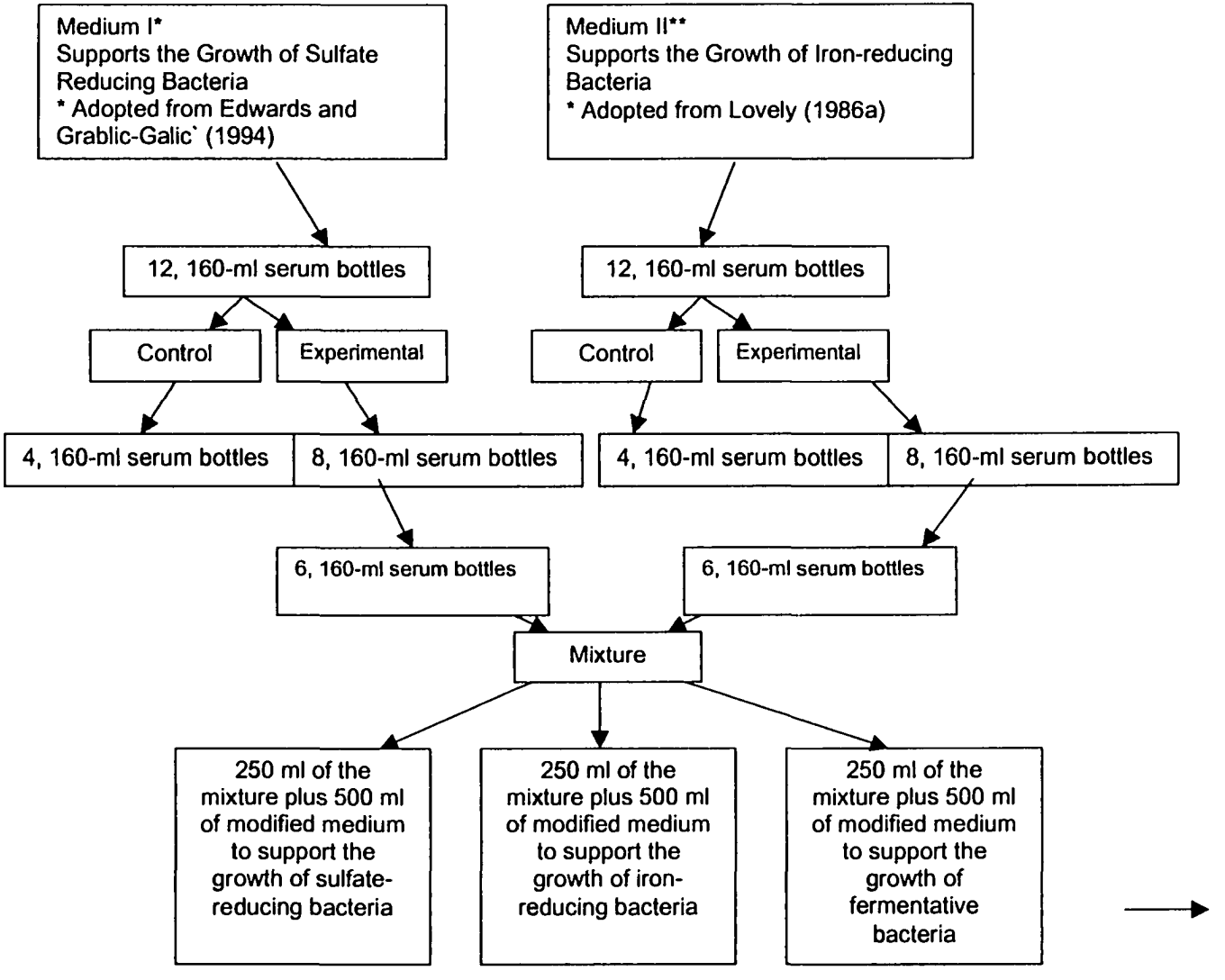
0.05 g/ l cyanocobalamin

0.05 g/l thioctic acid

APPENDIX F

EXPERIMENT SETUP

EXPERIMENT SETUP



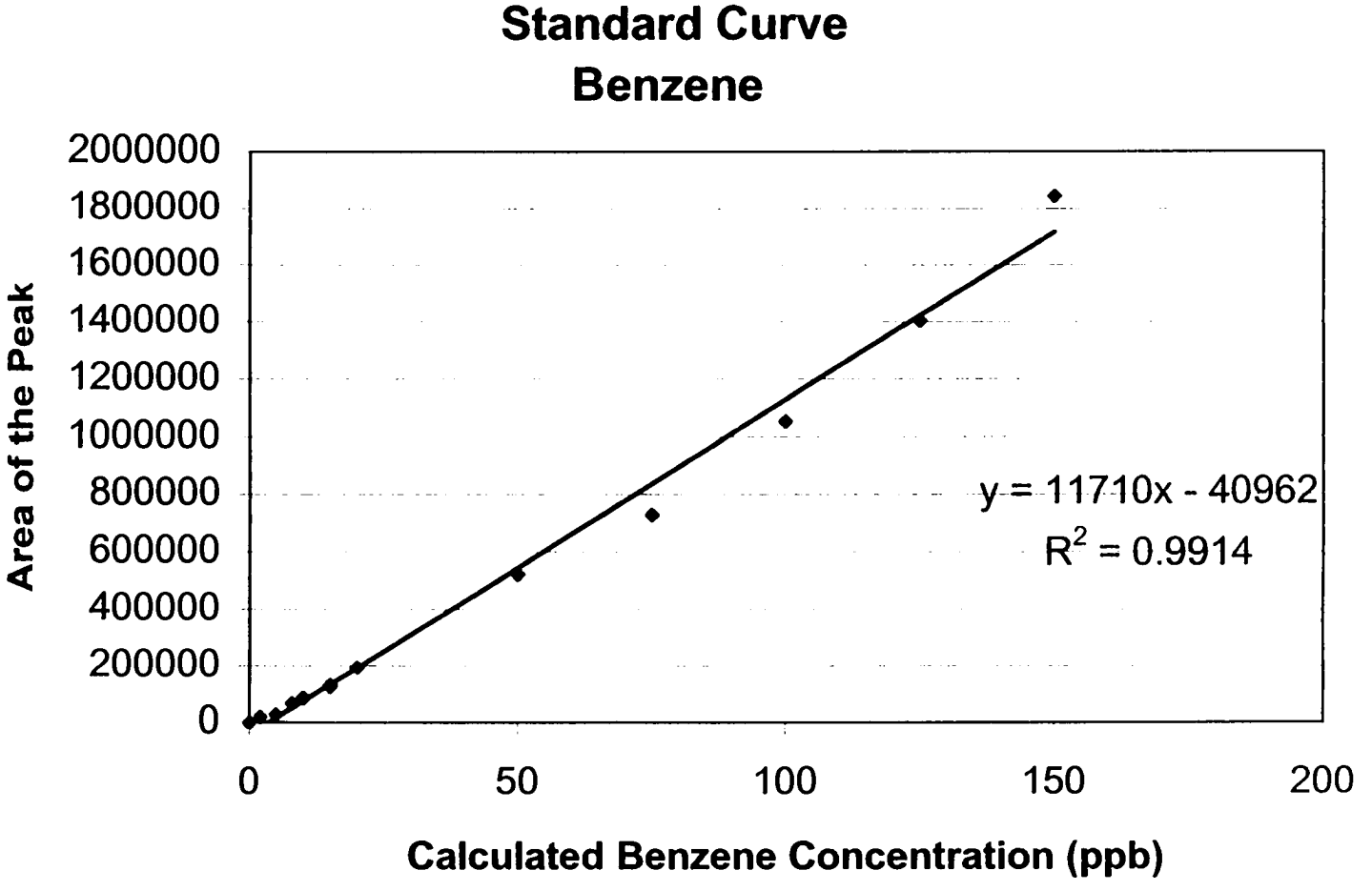
EXPERIMENT SETUP (Continued)

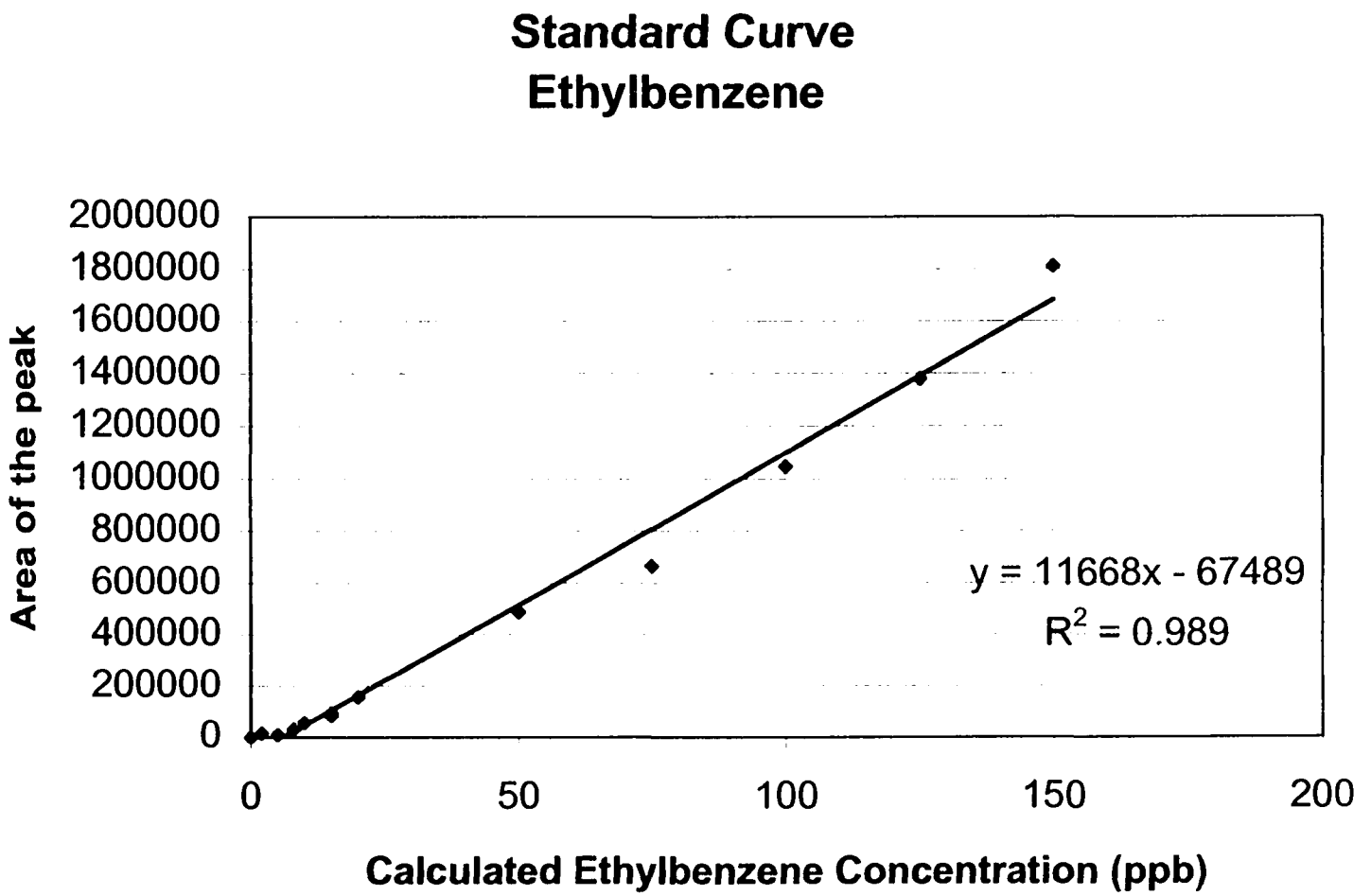
For each of the previous mixtures a set of 180, 30-ml serum bottles were prepared and incubated as follows:

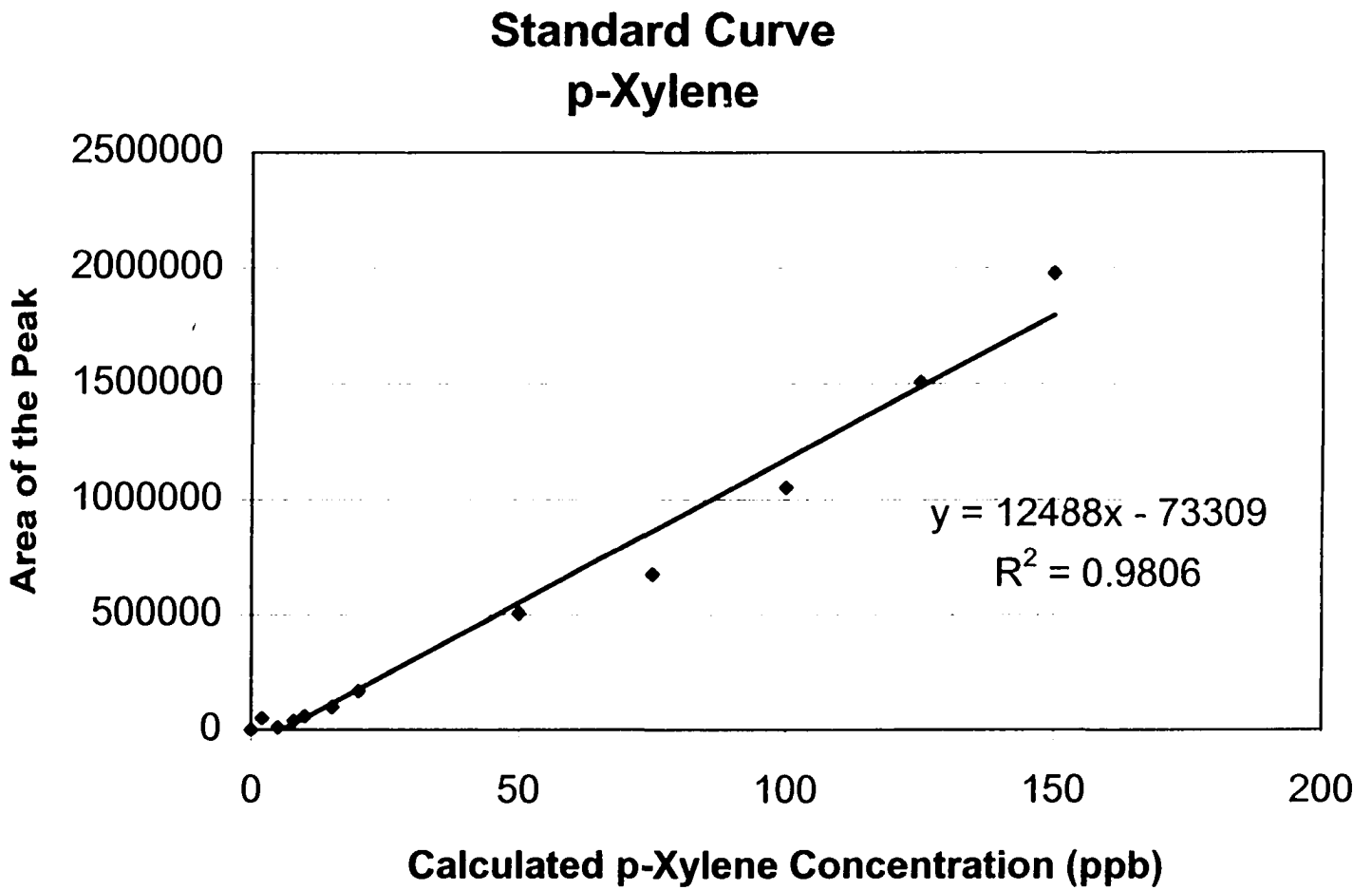
- 18, 30-ml serum bottles; 30 ml fresh medium/each+ 0.5mg/250 ml mercuric chloride/each bottle + 50 ppb benzene/each incubated at 21 °C
- 18, 30-ml serum bottles; 30 ml fresh medium/each + 0.5mg/250 ml mercuric chloride/each bottle + 50 ppb benzene/each incubated at 4 °C
- 18, 30-ml serum bottles; 30 ml fresh medium/each + autoclaved/each + 50 ppb benzene/each incubated at 21 °C
- 18, 30-ml serum bottles; 30 ml fresh medium/each + autoclaved/each + 50 ppb benzene/each incubated at 4 °C
- 18, 30-ml serum bottles; 30 ml fresh medium/each + 10 ppb benzene/each incubated at 21 °C
- 18, 30-ml serum bottles; 30 ml fresh medium/each + 10 ppb benzene/each incubated at 4 °C
- 18, 30-ml serum bottles; 30 ml fresh medium/each + 50 ppb benzene/each incubated at 21 °C
- 18, 30-ml serum bottles; 30 ml fresh medium/each + 50 ppb benzene/each incubated at 4 °C
- 18, 30-ml serum bottles; 30 ml fresh medium/each + 200 ppb benzene/each incubated at 21 °C
- 18, 30-ml serum bottles; 30 ml fresh medium/each + 200 ppb benzene/each incubated at 4 °C

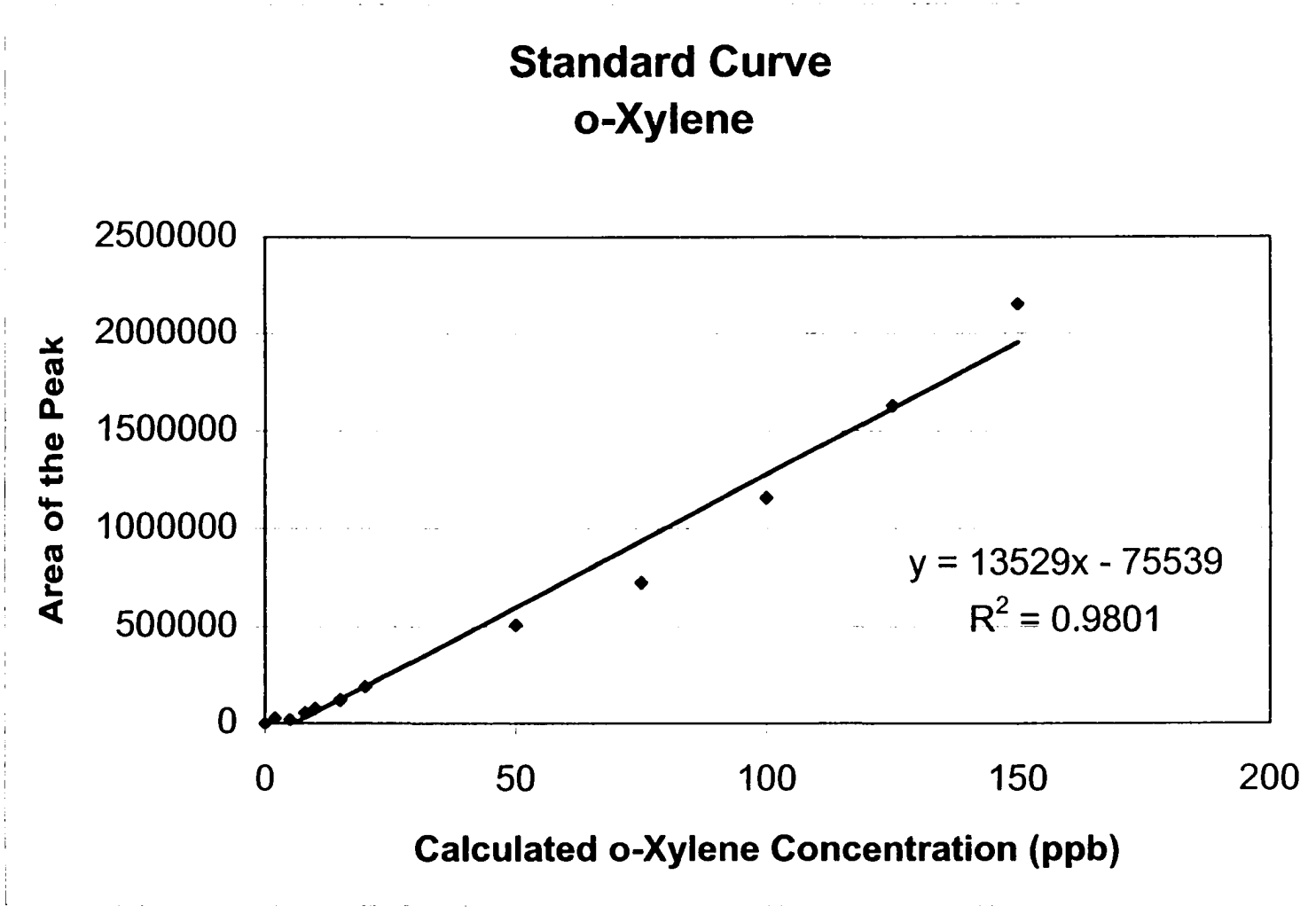
APPENDIX G

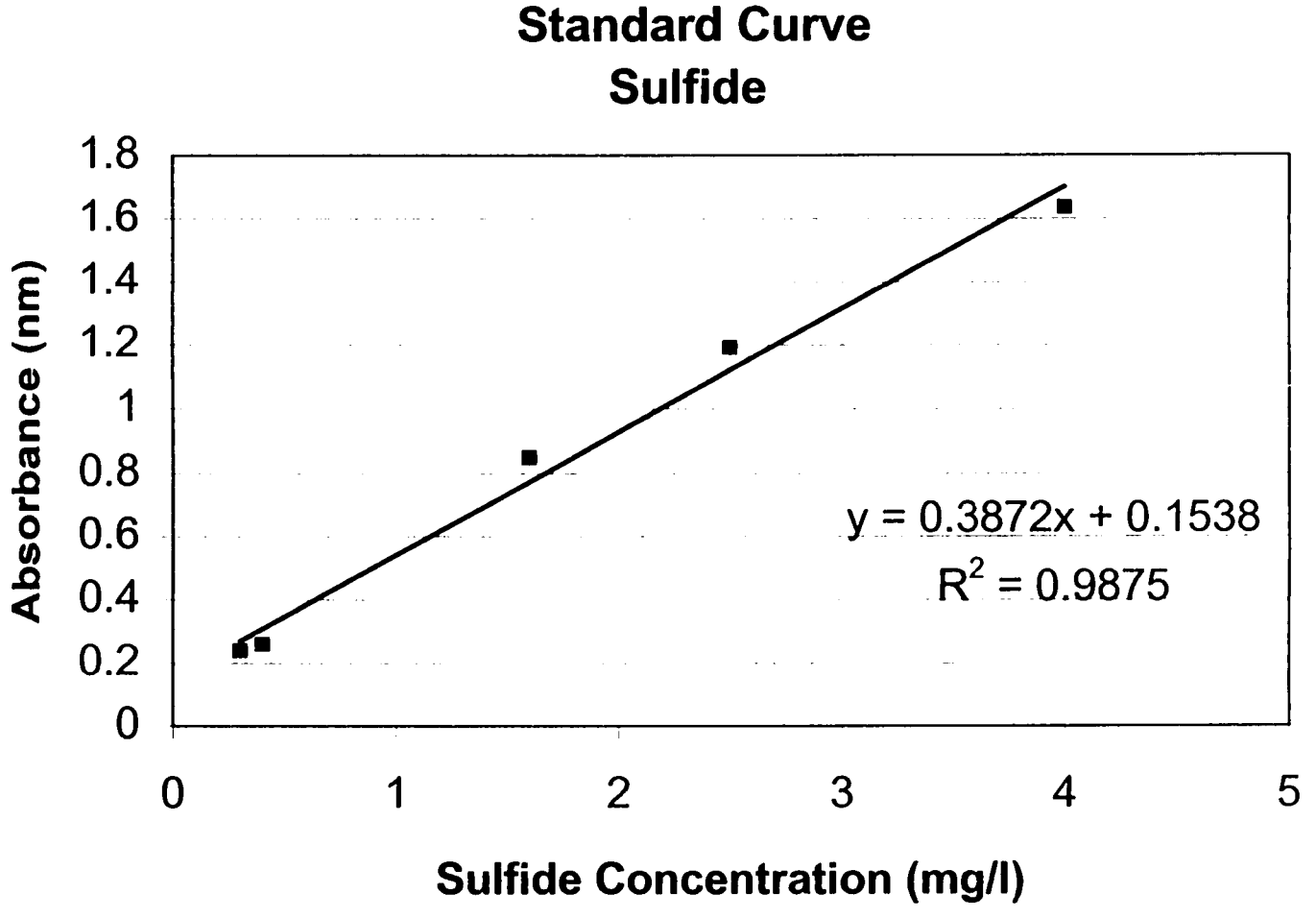
STANDARD CURVES

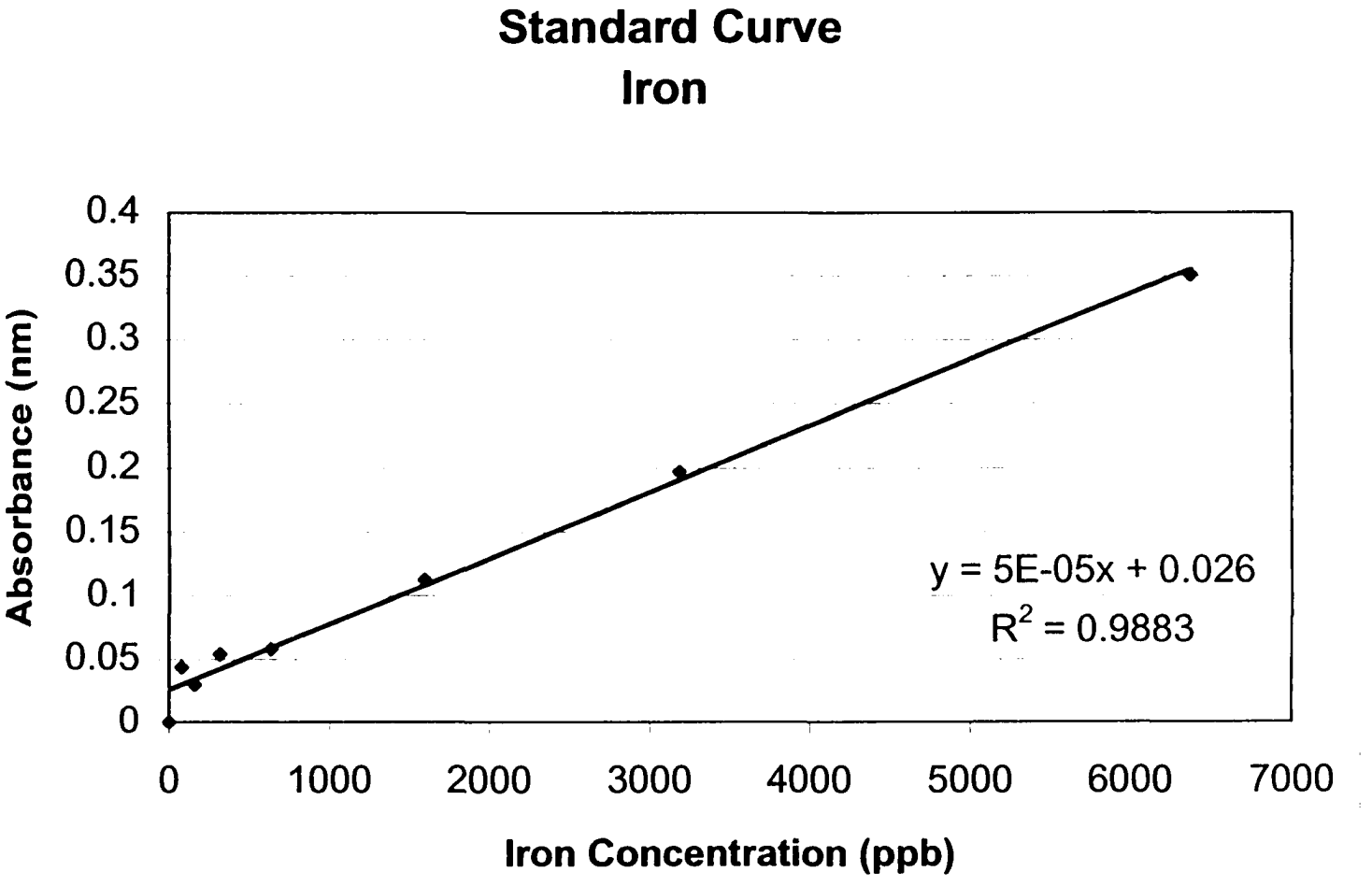


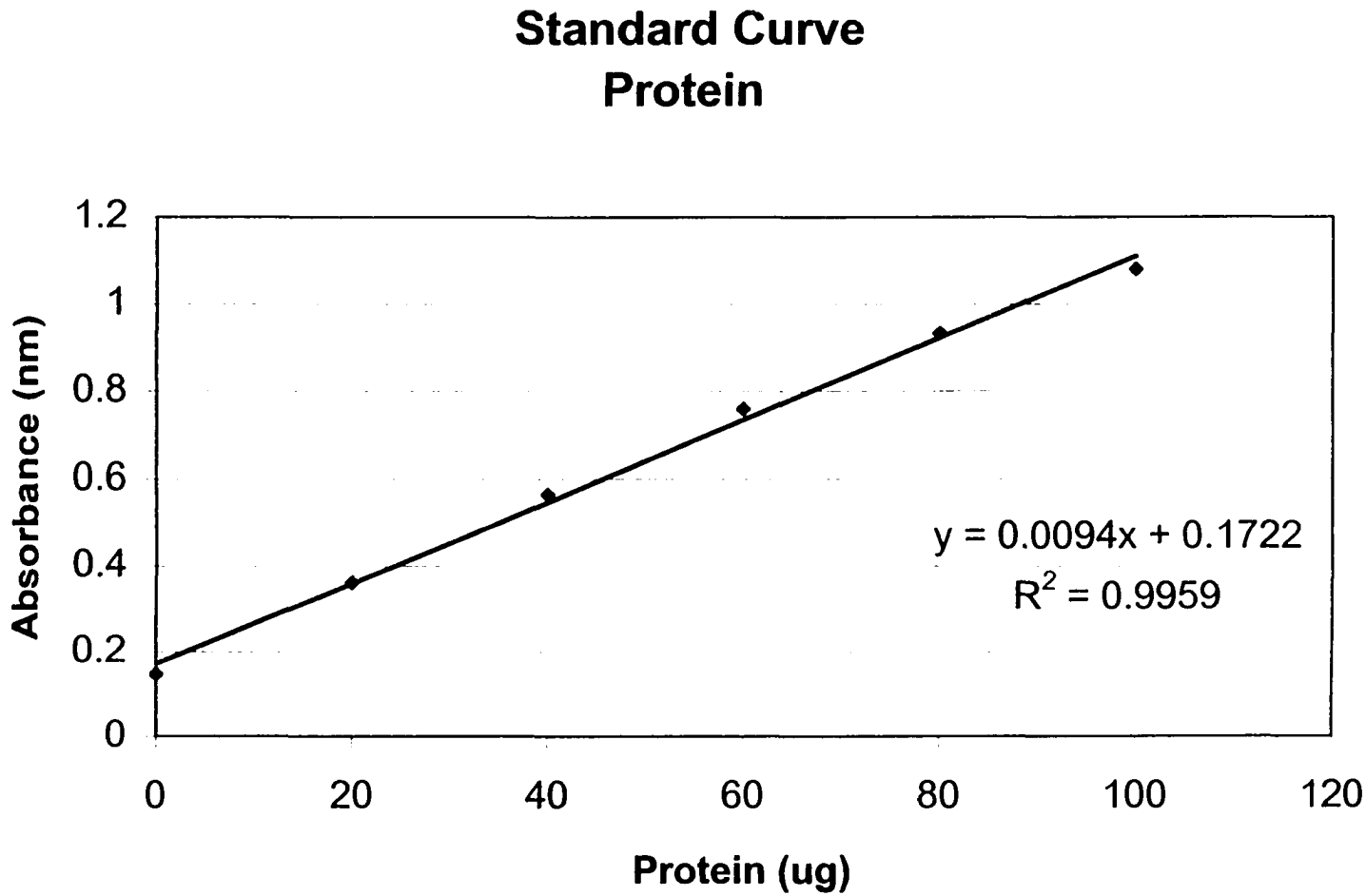












APPENDIX H

RESEARCH DATA FOR BENZENE DEGRADATION

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom v =(# of samples -1)

t.95 at v =1	6.31
t.95 at v =2	2.92
t.95 at v =3	2.35
t.95 at v =4	2.13

Iron-Reducing Bacteria
Benzene 50 ppb
Mercuric Chloride Control at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	77.81	60.81	14.80	30.25	91.36
1	50.87				
1	53.74				
2	67.56	67.56			
3	38.31	38.58	0.25	38.06	39.11
3	38.62				
3	38.82				
4	62.00	58.48	2.12	54.10	62.86
4	59.00				
4	54.45				
5	60.00	67.35	6.45	54.03	80.67
5	72.06				
5	70.00				
6	61.80	63.15	3.50	55.91	70.38
6	60.52				
6	67.13				

Iron-Reducing Bacteria
Benzene 50 ppb
Mercuric Chloride Control at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	79.58	77.58	3.65	70.04	85.13
1	73.37				
1	79.80				
2	55.67	55.67			
3	51.39	48.71	2.33	43.89	53.52
3	47.57				
3	47.16				
5	41.24	41.24			
6	74.87	73.77	1.57	63.86	83.67
6	72.66				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom *v* =(# of samples -1)

t.95 at v =1 6.31

t.95 at v =2 2.92

t.95 at v =3 2.35

t.95 at v =4 2.13

Iron-Reducing Bacteria
Benzene 50 ppb
Autoclaved Control at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
2	55.67	55.67		55.67	55.67
2					
2					
3	92.40	88.31	11.17	65.24	111.38
3	75.66				
3	96.85				
4	52.00	56.30	6.57	36.58	97.79
4	61.30				
4	55.61				
4	50.61				
5	52.00	50.59	4.51	41.27	59.90
5	45.54				
5	54.22				
6	65.91	67.70	3.15	61.19	74.22
6	65.85				
6	71.35				

Iron-Reducing Bacteria
Benzene 50 ppb
Autoclaved Control at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
2	67.27	74.35			
3	89.50	74.01	13.42	46.30	101.71
3	66.29				
3	66.23				
4	60.61	63.45	4.02	55.16	71.74
4	66.29				
4					
5	77.24	72.38	6.88	28.97	115.78
5	67.51				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom *v* =(# of samples -1)

t.95 at v =1	6.31
t.95 at v =2	2.92
t.95 at v =3	2.35
t.95 at v =4	2.13

Iron-Reducing Bacteria
Benzene 10 ppb
at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
	E				
1	[redacted]	6.77	4.38	0.00	15.81
1	3.89				
1	4.61				
2	1.64	3.15	1.35	0.36	5.93
2	3.57				
2	4.23				
3	3.46	3.46	0.00	3.46	3.46
3	3.46				
4	3.35	3.50	0.17	3.15	3.85
4	3.47				
4	3.68				
5	4.60	3.93	0.58	2.73	5.13
5	3.65				
5	3.53				
6	0.00	0.00	0.00	0.00	0.00
6	0.00				
6	0.00				

Iron-Reducing Bacteria
Benzene 10 ppb
at 4 °C

1	6.57	7.13	1.66	3.71	10.55
1	5.83				
1	[redacted]				
2	4.71	5.39	0.93	3.46	7.31
2	6.45				
2	5.01				
3	4.12	3.66	0.64	0.00	7.72
3	3.21				
4	3.56	3.69	0.19	3.30	4.08
4	3.61				
4	3.91				
5	4.83	5.53	1.22	3.01	8.04
5	4.81				
5	6.93			0.00	0.00
6	0.00	0.00			
6	0.00				
6	0.00				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom *v* =(# of samples -1)

t.95 at v =1	6.31
t.95 at v =2	2.92
t.95 at v =3	2.35
t.95 at v =4	2.13

Iron-Reducing Bacteria
Benzene 50 ppb
at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	4.01	3.87	0.20	2.63	5.11
1	3.73				
2	7.48	6.06	1.33	3.32	8.79
2	4.87				
2	5.82				
3	4.14	5.44	2.08	1.13	9.74
3	4.33				
3	7.84				
4	5.78	5.96	0.26	5.43	6.49
4	5.85				
4	6.26				
5	3.87	4.34	0.42	3.47	5.20
5	4.67				
5	4.47				
6	0.00	0.00			

Iron-Reducing Bacteria
Benzene 50 ppb
at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	[illegible]	33.23	34.67	0.00	251.98
1	8.71				
2	4.21	4.41	0.27	3.18	5.63
2	4.60				
3	3.13	3.25	0.16	2.53	3.96
3	3.36				
4	3.70	4.07	0.35	3.35	4.79
4	4.11				
4	4.39				
5	0.00	0.00	0.00	0.00	0.00
5	0.00				
5	0.00				
6	0.00	0.00	0.00	0.00	0.00
6	0.00				
6	0.00				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom *v* =(# of samples -1)

t.95 at v =1	6.31
t.95 at v =2	2.92
t.95 at v =3	2.35
t.95 at v =4	2.13

Iron-Reducing Bacteria
Benzene 200 ppb
at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	3.33	3.54	0.18	3.17	3.90
1	3.60				
1	3.67				
2	18.53	18.44	4.07	14.10	22.77
2	[redacted]				
2	[redacted]				
2	19.66				
2	4.20				
3	4.59	4.26	0.46	3.31	5.20
3	4.45				
3	3.73				
4	10.46	12.56	9.31	0.00	31.79
4	4.47				
4	22.74				
5	5.21	6.47	1.84	2.68	10.26
5	5.63				
5	8.58				
6	3.91	3.91	0.44	3.02	4.81
6	4.22				
6	3.61				

Iron-Reducing Bacteria
Benzene 200 ppb
at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	[redacted]	26.22	32.17	0.00	92.64
1	9.01				
1	6.31				
2	4.83	6.30	1.77	2.64	9.96
2	5.80				
2	8.26				
3	3.81	9.00	9.23	0.00	28.05
3	3.55				
3	19.65				
4	5.34	4.18	1.01	2.09	6.26
4	3.60				
4	3.58				
5	8.34	8.34			
6	3.79	5.44	2.34	0.00	20.19
6	7.09				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom v =(# of samples -1)

t.95 at v =1	6.31
t.95 at v =2	2.92
t.95 at v =3	2.35
t.95 at v =4	2.13

Sulfate-Reducing Bacteria
Benzene 50 ppb
Mercuric Chloride Control at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	58.78	57.43	2.90	51.45	63.41
1	59.39				
1	54.10				
2	52.66				
3	42.82				
4	54.00	57.08	4.35	29.63	84.52
4	60.15				
5	52.51	52.51			
6	52.51	52.51			

Sulfate-Reducing Bacteria
Benzene 50 ppb
Mercuric Chloride Control at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
2	59.40	61.70	3.25	41.17	82.23
2	64.00				
3	43.76	43.42	0.48	40.42	46.43
3	43.09				
4	68.67	60.54	2.96	54.42	66.66
4	64.48				
4	48.48	42.32			
5	42.32				
6	54.11	49.33	6.07	36.80	61.86
6	42.50				
6	51.38				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom *v* =(# of samples -1)

t.95 at v =1	6.31
t.95 at v =2	2.92
t.95 at v =3	2.35
t.95 at v =4	2.13

Sulfate-Reducing Bacteria
Benzene 50 ppb
Autoclaved Control at Room Tempertaure

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
2	81.28	81.28			
3	71.02	54.87	22.84	0.00	198.97
3	38.72				
4	56.87	55.34	1.72	51.79	58.90
4	53.48				
4	55.68				
5	43.99	56.76	18.06	0.00	170.71
5	69.53				
6	61.33	61.33			

Sulfate-Reducing Bacteria
Benzene 50 ppb
Autoclaved Control at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
2	39.14	39.14			
3	67.48	59.29	7.10	44.64	73.95
3	54.77				
3	55.64				
4	64.18	55.35	10.53	33.60	77.09
4	58.00				
4	59.13				
5	40.07	46.07	3.26	39.33	52.80
5	44.68				
5	53.46				
6	47.68	57.34	9.42	37.88	76.79
6	57.81				
6	66.51				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom *v* =(# of samples -1)

t.95 at v =1	6.31
t.95 at v =2	2.92
t.95 at v =3	2.35
t.95 at v =4	2.13

Sulfate-Reducing Bacteria
Benzene 10 ppb
at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	[redacted]	11.97	8.62	0.00	29.76
1	[redacted]				
1	3.13				
2	5.71	5.28	0.86	3.49	7.06
2	4.28				
2	5.84				
3	4.28	4.35	0.40	3.51	5.18
3	3.98				
3	4.78				
4	3.84	3.75	0.12	3.50	4.01
4	3.61				
4	3.81				
6	5.00	4.33	0.75	2.78	5.88
6	4.48				
6	3.52				

Sulfate-Reducing Bacteria
Benzene 10 ppb
at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	[redacted]	7.47	3.29	0.68	14.25
1	6.06				
1	5.12				
2	[redacted]	8.31	4.27	0.00	17.13
2	[redacted]				
2	3.38				
3	3.37	3.49	0.28	2.91	4.08
3	3.82				
3	3.29				
4	3.66	3.60	0.09	3.02	4.17
4	3.53				
5	3.40	3.39	2.26	-1.28	8.06
5	3.51				
5	3.27				
6	0.00	0.00	0.00	0.00	0.00
6	0.00				
6	0.00				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom *v* =(# of samples -1)

t.95 at v =1	6.31
t.95 at v =2	2.92
t.95 at v =3	2.35
t.95 at v =4	2.13

Sulfate-Reducing Bacteria
Benzene 50 ppb
at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	[illegible]	11.00	13.62	0.00	39.12
1	3.13				
1	3.13				
2	12.09	8.56	3.65	1.02	16.10
2	4.80				
2	8.79				
3	3.59	3.97	0.54	0.57	7.37
3	4.35				
4	3.38	3.47	0.08	3.30	3.65
4	3.50				
4	3.54				
5	3.38	3.46	0.12	3.21	3.71
5	3.54				
5					
6	0.00	0.00	0.00	0.00	0.00
6	0.00				
6	0.00				

Sulfate-Reducing Bacteria
Benzene 50 ppb
at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	12.46	15.80	4.92	5.64	25.96
1	[illegible]				
1	13.49				
2	3.80	3.74	0.06	3.62	3.86
2	3.68				
2	3.74				
3	7.52	6.28	2.06	0.00	15.49
3	3.89				
3	7.42				
4	5.63	4.69	1.33	0.00	13.07
4	3.75				
5	5.22	1.74	3.01	0.00	7.96
5	0.00				
5	0.00				
6	0.00				
6	0.00	0.00	0.00	0.00	0.00
6	0.00				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom *v* =(# of samples -1)

t.95 at v =1	6.31
t.95 at v =2	2.92
t.95 at v =3	2.35
t.95 at v =4	2.13

Sulfate-Reducing Bacteria
Benzene 200 ppb
at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	12.36	7.05	4.66	0.00	16.68
1	5.18				
1	3.61				
2	5.17	11.65	12.22	0.00	36.89
2	4.03				
2	[redacted]				
3	14.60	8.14	5.63	0.00	19.77
3	5.55				
3	4.27				
4	10.96	10.80	2.63	5.38	16.22
4	13.34				
4	8.10				
5	4.76	4.05	0.73	2.53	5.56
5	4.10				
5	3.29				
6	0.00	4.57	7.92	0.00	20.92
6	0.00				
6	13.71				

Sulfate-Reducing Bacteria
Benzene 200 ppb
at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	16.72	16.89	3.46	9.73	24.04
1	[redacted]				
1	13.51				
2	10.91	11.77	1.53	8.61	14.93
2	10.86				
2	13.54				
3	12.99	12.59	0.47	11.63	13.55
3	12.70				
3	12.08				
4	12.95	7.23	4.96	0.00	17.47
4	4.14				
4	4.60				
5	3.61	5.73	7.03	0.00	20.25
5	0.00				
5	13.57				
6	20.00	12.53	8.29	0.00	29.64
6	3.61				
6	13.98				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom *v* =(# of samples -1)

t.95 at v =1	6.31
t.95 at v =2	2.92
t.95 at v =3	2.35
t.95 at v =4	2.13

Fermentation and Methanogenesis

Benzene 50 ppb

Mercuric Chloride Control at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	48.58	48.60	3.86	40.63	56.57
1	52.47				
1	44.75				
3	46.23	46.61	2.66	41.12	52.10
3	44.16				
3	49.44				
4	35.72	40.21	4.10	31.75	48.67
4	43.74				
4	41.17				
5	47.22	47.96	0.85	46.20	49.71
5	48.89				
5	47.76				
6	36.43	39.75	3.67	32.17	47.33
6	39.14				
6	43.69				

Fermentation and Methanogenesis

Benzene 50 ppb

Mercuric Chloride Control at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	79.87	61.06	26.60	6.14	115.99
1					
1	42.25				
3	64.79	64.09	2.25	59.45	68.73
3	65.90				
3	61.57				
4	64.27	60.67	5.08	28.60	92.75
4	57.08				
5	63.62	61.83	2.20	57.28	66.37
5	60.51				
5	61.35				
6	58.19	52.48	10.43	30.95	74.00
6	58.79				
6	40.44				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom *v* =(# of samples -1)

t.95 at v =1	6.31
t.95 at v =2	2.92
t.95 at v =3	2.35
t.95 at v =4	2.13

Fermentation and Methanogenesis

Benzene 50 ppb

Autoclaved Control at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1					
1					
1					
2	54.57	50.76	8.74	32.71	68.81
2	56.95				
2	40.76				
3	55.20	52.84	10.38	31.40	74.28
3	41.48				
3	61.84				
4		46.81	13.99	17.91	75.70
4	36.91				
4	56.70				
5	38.72	41.31	3.21	34.68	47.94
5	44.90				
5	40.31				
6	57.70	51.16	8.54	33.53	68.79
6	54.29				
6	41.50				

Fermentation and Methanogenesis

Benzene 50 ppb

Autoclaved Control at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1					
1					
1					
2	35.28	40.70	7.67	24.87	56.53
2	46.12				
2					
3	58.23	56.61	12.20	31.42	81.80
3	67.92				
3	43.68				
4	45.06	46.05	2.79	40.29	51.81
4	43.89				
4	49.20				
5	52.74	60.82	7.72	44.88	76.77
5	63.66				
5	66.08				
6	46.00	42.12	3.88	34.11	50.13
6	42.12				
6	38.24				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom *v* =(# of samples -1)

t.95 at v =1	6.31
t.95 at v =2	2.92
t.95 at v =3	2.35
t.95 at v =4	2.13

Fermentation and Methanogenesis

Benzene 10 ppb

at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	[redacted]	11.20	2.62	0.00	36.13
1	[redacted]				
1	8.20				
2	[redacted]	8.10	1.91	4.15	12.04
2	6.54				
2	7.52				
3	4.30	3.97	0.29	3.37	4.56
3	3.80				
3	3.80				
4	5.10	4.70	0.87	2.90	6.50
4	5.30				
4	3.70				
5	3.67	4.10	0.69	2.68	5.52
5	4.89				
5	3.73				
6	4.40	3.97	0.40	3.13	4.80
6	3.60				
6	3.90				

Fermentation and Methanogenesis

Benzene 10 ppb

at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	4.60	4.60			
2	4.30	4.30			
3	3.69	3.92	2.27	0.00	18.25
3	4.14				
4	0.00	0.00	0.00	0.00	0.00
4	0.00				
4	0.00				
6	0.00	0.00	0.00	0.00	0.00
6	0.00				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom v =(# of samples -1)

t.95 at v =1	6.31
t.95 at v =2	2.92
t.95 at v =3	2.35
t.95 at v =4	2.13

Fermentation and Methanogenesis

Benzene 50 ppb

at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	5.62	19.51	24.42	0.00	69.92
1	5.20				
1	[redacted]				
2	0.00	3.68	3.62	0.00	11.14
2	7.23				
2	3.80				
3	4.10	4.06	0.17	3.83	4.29
3	3.87				
3	4.20				
3	4.10				
4	4.22	4.63	0.58	3.43	5.84
4	4.38				
4	5.30				
5	4.58	4.60	0.19	4.21	4.99
5	4.43				
5	4.80				
6	3.87	4.07	0.58	2.86	5.27
6	4.72				
6	3.61				

Fermentation and Methanogenesis

Benzene 50 ppb

at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	4.51	7.55	3.09		
1	7.46				
1	10.68				
2	8.09	9.76	2.88	0.00	27.91
2	11.42				
3	5.69	6.09	0.82	4.41	7.77
3	6.49				
3	7.32				
4	3.89	3.84	0.04	3.75	3.93
4	3.81				
4	3.82				
6	9.91	7.60	2.46	2.53	12.67
6	7.87				
6	5.02				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

RESEARCH DATA FOR BENZENE DEGRADATION

Small Sampling Theory

Mean -/+ t(Confidence Coefficients)/(Standard Deviation/# of samples-1)^0.5

# of samples 3 or 2

90% Confidence

Degree of Freedom *v* =(# of samples -1)

t.95 at v =1 6.31

t.95 at v =2 2.92

t.95 at v =3 2.35

t.95 at v =4 2.13

Fermentation and Methanogenesis

Benzene 200 ppb

at Room Temperature

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	11.20	70.44	100.84	0.00	278.64
1	13.24				
1	[redacted]				
2	3.83	5.65	3.19	0.00	12.23
2	3.79				
2	9.33				
3	5.83	5.43	0.57	4.24	6.61
3	5.02				
3	4.13				
4	4.22	7.04	2.45	1.99	12.09
4	8.54				
4	8.37				
5	0.00	0.00	0.00	0.00	0.00
5	0.00				
5	0.00				
6	5.40	6.32	2.13	1.91	10.73
6	4.80				
6	8.76				

Fermentation and Methanogenesis

Benzene 200 ppb

at 4 °C

Month	Benzene (ppb)	Mean	Std. Deviation	Confidence Limits	
1	6.45	8.35	9.44	0.00	27.84
1	18.59				
1	0.00				
2	8.27	20.22	24.50	0.00	70.81
2	[redacted]				
2	3.98				
3	3.72	2.49	2.15	0.00	6.93
3	3.74				
3	0.00				
4	4.62	7.69	3.14	1.20	14.18
4	7.55				
4	10.90				
5	4.75	4.72	0.45	3.79	5.64
5	5.15				
5	4.25				
6	12.34	8.75	3.12	2.30	15.19
6	7.20				
6	6.70				

*For the purpose of simplicity any number <0 is zero in the confidence limits values

APPENDIX I

CONCENTRATION OF BENZENE IN THE HEAD SPACE CALCULATED USING HENRY'S LAW CONSTANT

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ K=Hc/RT

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K
Limits +	0.25	
Limits -	-0.25	

Iron-Reducing Bacteria
Benzene 50 ppb
Mercuric Chloride Control at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	77.81	14.58	0.01
1	50.87	9.53	0.01
1	53.74	10.07	0.01
2	67.56	12.66	0.01
3	38.31	7.18	0.01
3	38.62	7.24	0.01
3	38.82	7.27	0.01
4	62.00	11.62	0.01
4	59.00	11.06	0.01
4	54.45	10.20	0.01
5	60.00	11.24	0.01
5	72.06	13.50	0.01
5	70.00	13.12	0.01
6	61.80	11.58	0.01
6	60.52	11.34	0.01
6	67.13	12.58	0.01

Iron-Reducing Bacteria
Benzene 50 ppb
Mercuric Chloride Control at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	79.58	5.84	0.00
1	73.37	5.38	0.00
1	79.80	5.86	0.00
2	55.67	4.09	0.00
3	51.39	3.77	0.00
3	47.57	3.49	0.00
3	47.16	3.46	0.00
5	41.24	3.03	0.00
6	74.87	5.49	0.00
6	72.66	5.33	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ K=Hc/RT

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Iron-Reducing Bacteria
Benzene 50 ppb
Autoclaved Control at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
2	55.67	10.43	0.01
2			
2			
3	92.40	17.31	0.01
3	75.66	14.18	0.01
3	96.85	18.15	0.01
4	52.00	9.74	0.01
4	61.30	11.49	0.01
4	55.61	10.42	0.01
4	50.61	9.48	0.01
5	52.00	9.74	0.01
5	45.54	8.53	0.01
5	54.22	10.16	0.01
6	65.91	12.35	0.01
6	65.85	12.34	0.01
6	71.35	13.37	0.01

Iron-Reducing Bacteria
Benzene 50 ppb
Autoclaved Control at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
2	67.27	4.94	0.00
3	89.50	6.57	0.00
3	66.29	4.86	0.00
3	66.23	4.86	0.00
4	60.61	4.45	0.00
4	66.29	4.86	0.00
4			
5	77.24	5.67	0.00
5	67.51	4.95	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ $K = Hc/RT$

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Iron-Reducing Bacteria
Benzene 10 ppb
at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	11.81	2.21	0.01
1	3.89	0.73	0.01
1	4.61	0.86	0.01
2	1.64	0.31	0.01
2	3.57	0.67	0.01
2	4.23	0.79	0.01
3	3.46	0.65	0.01
3	3.46	0.65	0.01
4	3.35	0.63	0.01
4	3.47	0.65	0.01
4	3.68	0.69	0.01
5	4.60	0.86	0.01
5	3.65	0.68	0.01
5	3.53	0.66	0.01
6	0.00	0.00	0.00
6	0.00	0.00	0.00
6	0.00	0.00	0.00

Iron-Reducing Bacteria
Benzene 10 ppb
at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	6.57	0.48	0.00
1	5.83	0.43	0.00
1	9.00	0.66	0.00
2	4.71	0.35	0.00
2	6.45	0.47	0.00
2	5.01	0.37	0.00
3	4.12	0.30	0.00
3	3.21	0.24	0.00
4	3.56	0.26	0.00
4	3.61	0.26	0.00
4	3.91	0.29	0.00
5	4.83	0.35	0.00
5	4.81	0.35	0.00
5	6.93	0.51	0.00
6	0.00	0.00	0.00
6	0.00	0.00	0.00
6	0.00	0.00	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ K=Hc/RT

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Iron-Reducing Bacteria
Benzene 50 ppb
at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	4.01	0.75	0.01
1	3.73	0.70	0.01
2	7.48	1.40	0.01
2	4.87	0.91	0.01
2	5.82	1.09	0.01
3	4.14	0.78	0.01
3	4.33	0.81	0.01
3	7.84	1.47	0.01
4	5.78	1.08	0.01
4	5.85	1.10	0.01
4	6.26	1.17	0.01
5	3.87	0.72	0.01
5	4.67	0.88	0.01
5	4.47	0.84	0.01
6	0.00	0.00	0.00

Iron-Reducing Bacteria
Benzene 50 ppb
at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	57.74	4.24	0.00
1	8.71	0.64	0.00
2	4.21	0.31	0.00
2	4.60	0.34	0.00
3	3.13	0.23	0.00
3	3.36	0.25	0.00
4	3.70	0.27	0.00
4	4.11	0.30	0.00
4	4.39	0.32	0.00
5	0.00	0.00	0.00
5	0.00	0.00	0.00
5	0.00	0.00	0.00
6	0.00	0.00	0.00
6	0.00	0.00	0.00
6	0.00	0.00	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ $K = Hc/RT$

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Iron-Reducing Bacteria
Benzene 200 ppb
at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	3.33	0.62	0.00
1	3.60	0.68	0.00
1	3.67	0.69	0.00
2	18.53	3.47	0.00
2	23.13	4.33	0.00
2	26.66	4.99	0.00
2	19.66	3.68	0.00
2	4.20	0.79	0.00
3	4.59	0.86	0.00
3	4.45	0.83	0.00
3	3.73	0.70	0.00
4	10.46	1.96	0.00
4	4.47	0.84	0.00
4	22.74	4.26	0.00
5	5.21	0.98	0.00
5	5.63	1.05	0.00
5	8.58	1.61	0.00
6	3.91	0.73	0.00
6	4.22	0.79	0.00
6	3.61	0.68	0.00

Iron-Reducing Bacteria
Benzene 200 ppb
at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	63.33	4.65	0.00
1	9.01	0.66	0.00
1	6.31	0.46	0.00
2	4.83	0.35	0.00
2	5.80	0.43	0.00
2	8.26	0.61	0.00
3	3.81	0.28	0.00
3	3.55	0.26	0.00
3	19.65	1.44	0.00
4	5.34	0.39	0.00
4	3.60	0.26	0.00
4	3.58	0.26	0.00
5	8.34	0.61	0.00
6	3.79	0.28	0.00
6	7.09	0.52	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ $K=Hc/RT$

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Sulfate-Reducing Bacteria
Benzene 50 ppb
Mercuric Chloride Control at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	58.78	11.01	0.00
1	59.39	11.13	0.00
1	54.10	10.14	0.00
2	52.66	9.87	0.00
3	42.82	8.02	0.00
4	54.00	10.12	0.00
4	60.15	11.27	0.00
5	52.51	9.84	0.00
6	52.51	9.84	0.00

Sulfate-Reducing Bacteria
Benzene 50 ppb
Mercuric Chloride Control at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
2	59.40	4.36	0.00
2	64.00	4.70	0.00
3	43.76	3.21	0.00
3	43.09	3.16	0.00
4	68.67	5.04	0.00
4	64.48	4.73	0.00
4	48.48	3.56	0.00
5	42.32	3.11	0.00
6	54.11	3.97	0.00
6	42.50	3.12	0.00
6	51.38	3.77	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ K=Hc/RT

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Sulfate-Reducing Bacteria
Benzene 50 ppb
Autoclaved Control at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
2	81.28	15.23	0.00
3	71.02	13.31	0.00
3	38.72	7.26	0.00
4	56.87	10.66	0.00
4	53.48	10.02	0.00
4	55.68	10.43	0.00
5	43.99	8.24	0.00
5	69.53	13.03	0.00
6	61.33	11.49	0.00

Sulfate-Reducing Bacteria
Benzene 50 ppb
Autoclaved Control at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
2	39.14	2.87	0.00
3	67.48	4.95	0.00
3	54.77	4.02	0.00
3	55.64	4.08	0.00
4	64.18	4.71	0.00
4	58.00	4.26	0.00
4	59.13	4.34	0.00
5	40.07	2.94	0.00
5	44.68	3.28	0.00
5	53.46	3.92	0.00
6	47.68	3.50	0.00
6	57.81	4.24	0.00
6	66.51	4.88	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ $K = Hc/RT$

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Sulfate-Reducing Bacteria
Benzene 10 ppb
at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	20.35	3.81	0.00
1	12.41	2.33	0.00
1	3.13	0.59	0.00
2	5.71	1.07	0.00
2	4.28	0.80	0.00
2	5.84	1.09	0.00
3	4.28	0.80	0.00
3	3.98	0.75	0.00
3	4.78	0.90	0.00
4	3.84	0.72	0.00
4	3.61	0.68	0.00
4	3.81	0.71	0.00
6	5.00	0.94	0.00
6	4.48	0.84	0.00
6	3.52	0.66	0.00

Sulfate-Reducing Bacteria
Benzene 10 ppb
at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	11.22	0.82	0.00
1	6.06	0.44	0.00
1	5.12	0.38	0.00
2	10.71	0.79	0.00
2	10.84	0.80	0.00
2	3.38	0.25	0.00
3	3.37	0.25	0.00
3	3.82	0.28	0.00
3	3.29	0.24	0.00
4	3.66	0.27	0.00
4	3.53	0.26	0.00
5	3.40	0.25	0.00
5	3.51	0.26	0.00
5	3.27	0.24	0.00
6	0.00	0.00	0.00
6	0.00	0.00	0.00
6	0.00	0.00	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c_l/c_{aq}$ $K = Hc/RT$

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Sulfate-Reducing Bacteria
Benzene 50 ppb
at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	26.73	5.01	0.00
1	3.13	0.59	0.00
1	3.13	0.59	0.00
2	12.09	2.27	0.00
2	4.80	0.90	0.00
2	8.79	1.65	0.00
3	3.59	0.67	0.00
3	4.35	0.81	0.00
4	3.38	0.63	0.00
4	3.50	0.66	0.00
4	3.54	0.66	0.00
5	3.38	0.63	0.00
5	3.54	0.66	0.00
5		0.00	0.00
6	0.00	0.00	0.00
6	0.00	0.00	0.00
6	0.00	0.00	0.00

Sulfate-Reducing Bacteria
Benzene 50 ppb
at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	12.46	0.91	0.00
1	21.45	1.57	0.00
1	13.49	0.99	0.00
2	3.80	0.28	0.00
2	3.68	0.27	0.00
2	3.74	0.27	0.00
3	7.52	0.55	0.00
3	3.89	0.29	0.00
3	7.42	0.54	0.00
4	5.63	0.41	0.00
4	3.75	0.28	0.00
5	5.22	0.38	0.00
5	0.00	0.00	0.00
5	0.00	0.00	0.00
6	0.00	0.00	0.00
6	0.00	0.00	0.00
6	0.00	0.00	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ K=Hc/RT

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Sulfate-Reducing Bacteria
Benzene 200 ppb
at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	12.36	2.32	0.00
1	5.18	0.97	0.00
1	3.61	0.68	0.00
2	5.17	0.97	0.00
2	4.03	0.75	0.00
2	25.75	4.82	0.00
3	14.60	2.74	0.00
3	5.55	1.04	0.00
3	4.27	0.80	0.00
4	10.96	2.05	0.00
4	13.34	2.50	0.00
4	8.10	1.52	0.00
5	4.76	0.89	0.00
5	4.10	0.77	0.00
5	3.29	0.62	0.00
6	0.00	0.00	0.00
6	0.00	0.00	0.00
6	13.71	2.57	0.00

Sulfate-Reducing Bacteria
Benzene 200 ppb
at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	16.72	1.23	0.00
1	20.44	1.50	0.00
1	13.51	0.99	0.00
2	10.91	0.80	0.00
2	10.86	0.80	0.00
2	13.54	0.99	0.00
3	12.99	0.95	0.00
3	12.70	0.93	0.00
3	12.08	0.89	0.00
4	12.95	0.95	0.00
4	4.14	0.30	0.00
4	4.60	0.34	0.00
5	3.61	0.27	0.00
5	0.00	0.00	0.00
5	13.57	1.00	0.00
6	20.00	1.47	0.00
6	3.61	0.27	0.00
6	13.98	1.03	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ K=Hc/RT

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Fermentation and Methanogenesis
Benzene 50 ppb
Mercuric Chloride Control at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	48.58	9.10	0.00
1	52.47	9.83	0.00
1	44.75	8.38	0.00
3	46.23	8.66	0.00
3	44.16	8.27	0.00
3	49.44	9.26	0.00
4	35.72	6.69	0.00
4	43.74	8.20	0.00
4	41.17	7.71	0.00
5	47.22	8.85	0.00
5	48.89	9.16	0.00
5	47.76	8.95	0.00
6	36.43	6.83	0.00
6	39.14	7.33	0.00
6	43.69	8.19	0.00

Fermentation and Methanogenesis
Benzene 50 ppb
Mercuric Chloride Control at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	79.87	5.86	0.00
1	42.25	3.10	0.00
3	64.79	4.75	0.00
3	65.90	4.84	0.00
3	61.57	4.52	0.00
4	64.27	4.72	0.00
4	57.08	4.19	0.00
5	63.62	4.67	0.00
5	60.51	4.44	0.00
5	61.35	4.50	0.00
6	58.19	4.27	0.00
6	58.79	4.31	0.00
6	40.44	2.97	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ $K = Hc/RT$

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Fermentation and Methanogenesis
Benzene 50 ppb
Autoclaved Control at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1			
1			
1			
2	54.57	10.22	0.00
2	56.95	10.67	0.00
2	40.76	7.64	0.00
3	55.20	10.34	0.00
3	41.48	7.77	0.00
3	61.84	11.59	0.00
4	36.91	6.92	0.00
4	56.70	10.62	0.00
5	38.72	7.25	0.00
5	44.90	8.41	0.00
5	40.31	7.55	0.00
6	57.70	10.81	0.00
6	54.29	10.17	0.00
6	41.50	7.78	0.00

Fermentation and Methanogenesis
Benzene 50 ppb
Autoclaved Control at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
2	35.28	2.59	0.00
2	46.12	3.38	0.00
3	58.23	4.27	0.00
3	67.92	4.98	0.00
3	43.68	3.21	0.00
4	45.06	3.31	0.00
4	43.89	3.22	0.00
4	49.20	3.61	0.00
5	52.74	3.87	0.00
5	63.66	4.67	0.00
5	66.08	4.85	0.00
6	46.00	3.38	0.00
6	42.12	3.09	0.00
6	38.24	2.81	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ K=Hc/RT

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Fermentation and Methanogenesis
Benzene 10 ppb
at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	13.00	2.44	0.00
1	12.40	2.32	0.00
1	8.20	1.54	0.00
2	10.23	1.92	0.00
2	6.54	1.23	0.00
2	7.52	1.41	0.00
3	4.30	0.81	0.00
3	3.80	0.71	0.00
3	3.80	0.71	0.00
4	5.10	0.96	0.00
4	5.30	0.99	0.00
4	3.70	0.69	0.00
5	3.67	0.69	0.00
5	4.89	0.92	0.00
5	3.73	0.70	0.00
6	4.40	0.82	0.00
6	3.60	0.67	0.00
6	3.90	0.73	0.00

Fermentation and Methanogenesis
Benzene 10 ppb
at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	4.60	0.34	0.00
2	4.30	0.32	0.00
3	3.69	0.27	0.00
3	4.14	0.30	0.00
4	0.00	0.00	0.00
4	0.00	0.00	0.00
4	0.00	0.00	0.00
6	0.00	0.00	0.00
6	0.00	0.00	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ $K = Hc/RT$

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Fermentation and Methanogenesis
Benzene 50 ppb
at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	5.62	1.05	0.00
1	5.20	0.97	0.00
1	47.70	8.94	0.00
2	0.00	0.00	0.00
2	7.23	1.35	0.00
2	3.80	0.71	0.00
3	4.10	0.77	0.00
3	3.87	0.73	0.00
3	4.20	0.79	0.00
3	4.10	0.77	0.00
4	4.22	0.79	0.00
4	4.38	0.82	0.00
4	5.30	0.99	0.00
5	4.58	0.86	0.00
5	4.43	0.83	0.00
5	4.80	0.90	0.00
6	3.87	0.72	0.00
6	4.72	0.88	0.00
6	3.61	0.68	0.00

Fermentation and Methanogenesis
Benzene 50 ppb
at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	4.51	0.33	0.00
1	7.46	0.55	0.00
1	10.68	0.78	0.00
2	8.09	0.59	0.00
2	11.42	0.84	0.00
3	5.69	0.42	0.00
3	6.49	0.48	0.00
3	7.32	0.54	0.00
4	3.89	0.29	0.00
4	3.81	0.28	0.00
4	3.82	0.28	0.00
6	9.91	0.73	0.00
6	7.87	0.58	0.00
6	5.02	0.37	0.00

Concentration of Benzene in the Head Space Calculated using the Henry' s Law Constant

$K = c/c_{aq}$ K=Hc/RT

Item	Value	Units
R	8.31	Pa m^3/mol K
T at 21 °C	294.00	K
T at 4 °C	277.00	K
K constant 21 °C	0.19	dimensionless
K constant at 4 °C	0.07	dimensionless
H constant at 21 °C	458.00	Pa m^3/mol K
H constant at 4 °C	169.00	Pa m^3/mol K

Fermentation and Methanogenesis
Benzene 200 ppb
at 21 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	11.20	2.10	0.00
1	13.24	2.48	0.00
1	186.87	35.01	0.00
2	3.83	0.72	0.00
2	3.79	0.71	0.00
2	9.33	1.75	0.00
3	5.83	1.09	0.00
3	5.02	0.94	0.00
3	4.13	0.77	0.00
4	4.22	0.79	0.00
4	8.54	1.60	0.00
4	8.37	1.57	0.00
5	0.00	0.00	0.00
5	0.00	0.00	0.00
5	0.00	0.00	0.00
6	5.40	1.01	0.00
6	4.80	0.90	0.00
6	8.76	1.64	0.00

Fermentation and Methanogenesis
Benzene 200 ppb
at 4 °C

Month	Benzene Concentration in Aqueous Solution (ppb)	Benzene Concentration in Head Space (ppb)	Benzene Headspace Conc.* Volume/ Benzene Solution Conc.* Volume
1	6.45	0.47	0.00
1	18.59	1.36	0.00
1	0.00	0.00	0.00
2	8.27	0.61	0.00
2	48.40	3.55	0.00
2	3.98	0.29	0.00
3	3.72	0.27	0.00
3	3.74	0.27	0.00
3	0.00	0.00	0.00
4	4.62	0.34	0.00
4	7.55	0.55	0.00
4	10.90	0.80	0.00
5	4.75	0.35	0.00
5	5.15	0.38	0.00
5	4.25	0.31	0.00
6	12.34	0.91	0.00
6	7.20	0.53	0.00
6	6.70	0.49	0.00

APPENDIX J

PHOTOGRAPHS ILLUSTRATING THE RESEARCH METHODS

Figure J1: Set up for sampling from FWM 6894 at Fort Wainwright, Alaska

Figure J2: Glove bag purged with nitrogen gas while collecting contaminated groundwater samples in serum bottles

Figure J3: The bailer was purged with nitrogen gas while water well sampling was taking place.

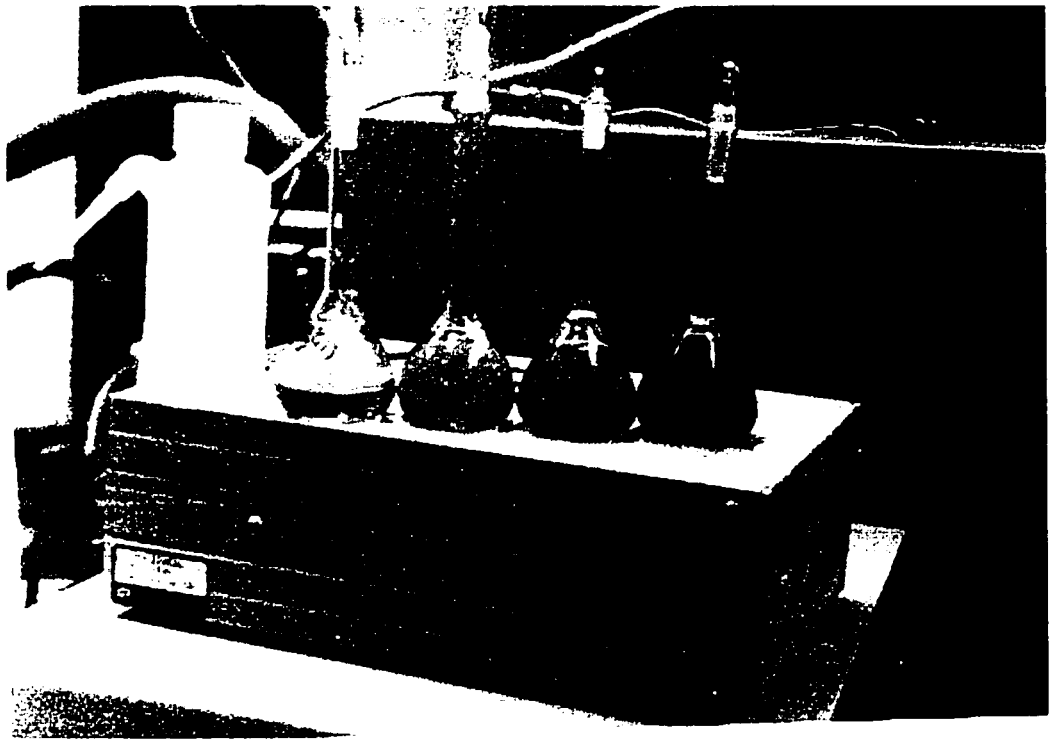


Figure J4: Iron standards were used to detect the presence of ferrous iron.

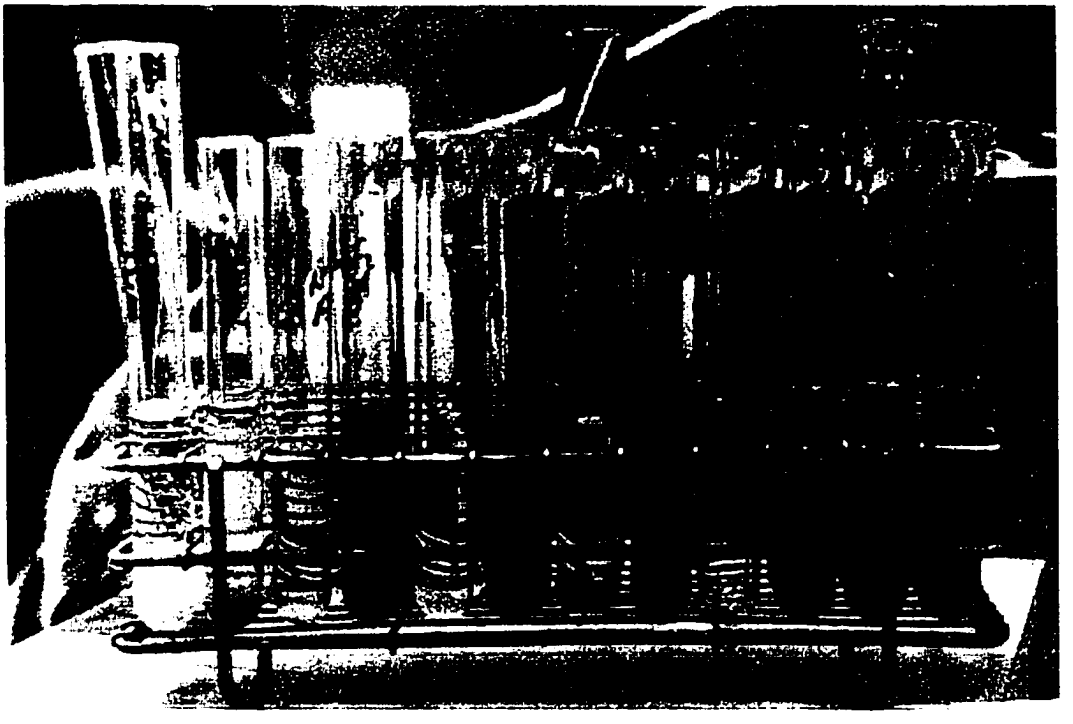
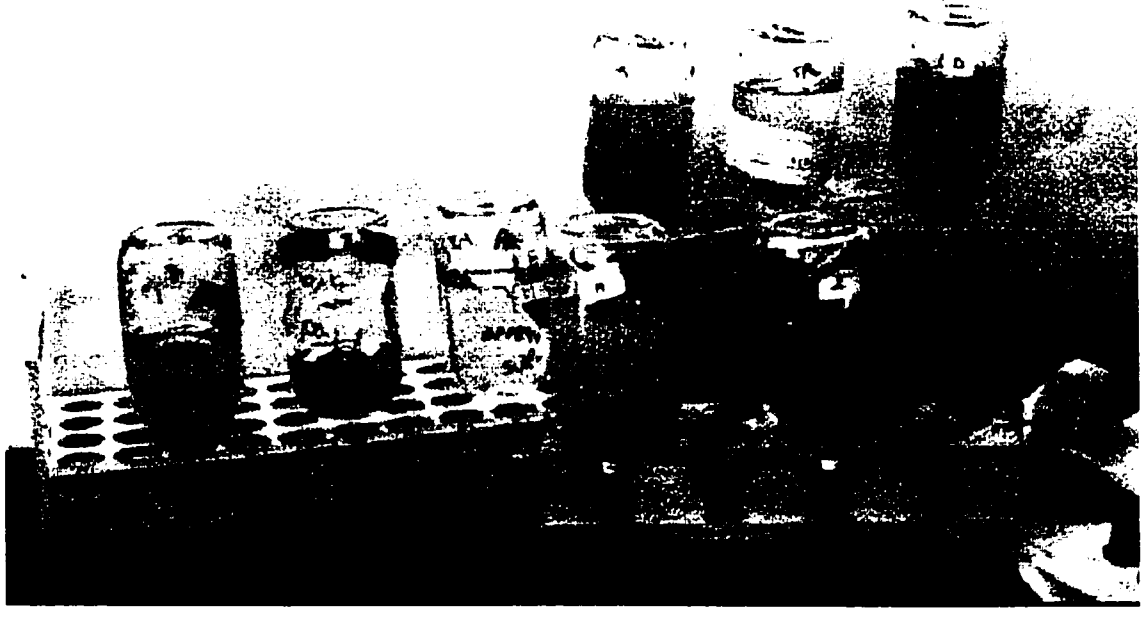


Figure J5: Samples in which the blue color indicates the presence of sulfide ions.

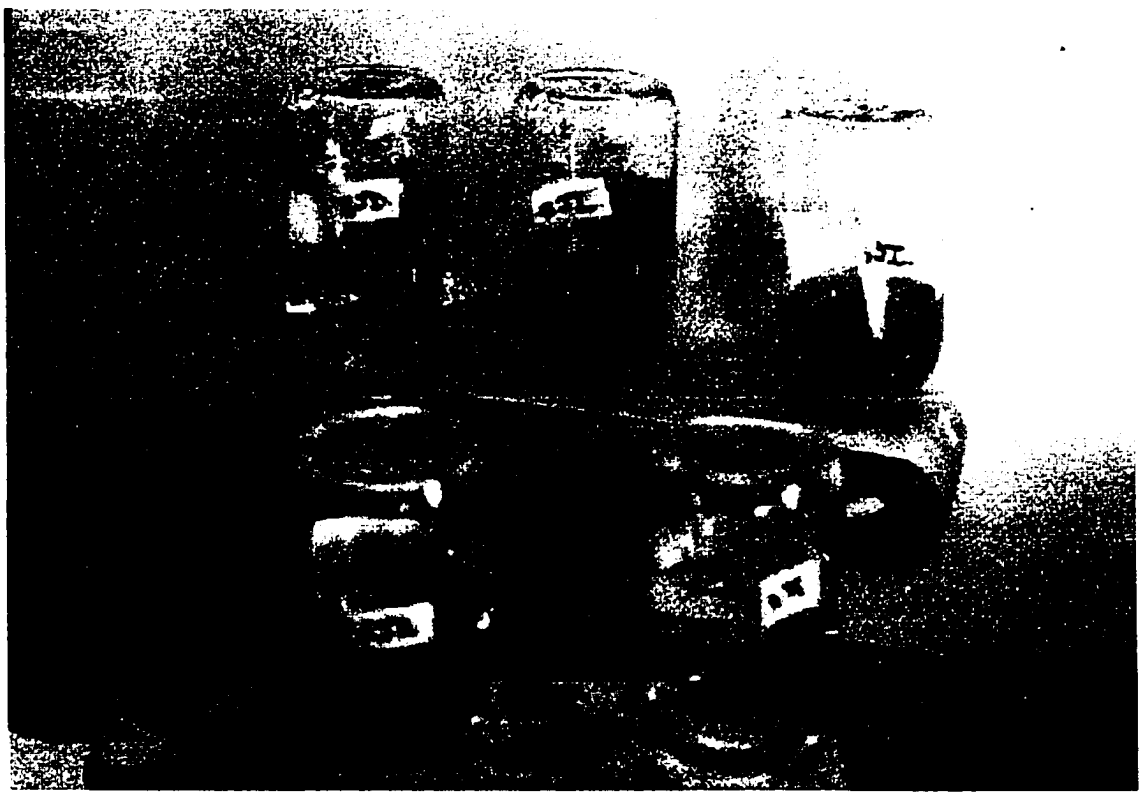


A

Figure J6: Serum bottles in which anaerobic conditions throughout the incubation period were confirmed by the clear color of the redox indicator, resazurin.

B

Figure J7: Serum bottles in which anaerobic conditions throughout the incubation period were confirmed by the clear color of the redox indicator, resazurin.



C

Figure J8: Serum bottlesin which anaerobic conditions through out the incubation period were confirmed by the clear color of the redox indicator, resazurin.